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METHOD OF IDENTIFYING INHIBITORS OF TIE-2

RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 60/192,920, filed on March 29, 2000. The entire teachings of the above application is incorporated herein by reference.

BACKGROUND OF THE INVENTION

Angiogenesis is a fundamental process by which new blood vessels are formed through sprouting, branching, proliferation, and tubule formation by endothelial cells from existing vasculature. In healthy humans, this neovascularization is under stringent control, normally occurring only during embryonic development, endometrial regulation, breast lactation and wound repair. However, in many pathological conditions, such as rheumatoid arthritis, solid tumors, Kaposi's sarcoma, blindness due to ocular neovascularization, psoriasis and atherosclerosis, disease progression is dependent upon persistent angiogenesis. The vasculature, which is the conduit for drug delivery, is one of the most accessible tissues in the body. Each endothelial cell of tumor vessels is estimated to support 100 to 1,000 neighboring cells, yet in the absence of an angiogenic stimulus endothelial cells typically divide only once every thousand days.

A number of polypeptide growth factors and their associated endothelial cell specific receptors have been discovered which are primarily responsible for the stimulation of endothelial cell growth, differentiation and the establishment of new

vasculature. These growth factor receptors include the vascular endothelial growth factor receptors (VEGFR) Flk-1 (mouse), KDR/VEG-FR-2 (human), Flt-1/VEGFR-1, and Flt-4/VEGFR-3. Receptors which are responsible for neovascularizaton also include the receptor tyrosine kinases Tie-1 and Tie-2.

Due to its role in regulating new vascular development, Tie-2 is a potential target for therapies aimed at controlling diseases which depend upon persistent angiogenesis. The development of biochemical assays for Tie-2 has enabled drug discovery to proceed along the pathways of identifying lead Tie-2 inhibitors by high-throughput screening of compound libraries and by testing compounds that mimic substrate structure; however, rational, structure-based design has not been possible up to this point because of the lack of accurate three-dimensional structural data for Tie-2 receptors.

SUMMARY OF THE INVENTION

The present invention relates to a polypeptide which comprises the catalytic domain of Tie-2, a crystalline form of this polypeptide and the use of structural information derived from the crystalline form of the polypeptide for designing and/or identifying potential inhibitors of the binding of one or more native ligands to the catalytic domain of Tie-2.

In one embodiment, the present invention relates to a polypeptide comprising the catalytic domain of TIE-2 and having the amino acid sequence set forth in SEQ ID NO: 2. In another embodiment, the invention relates to a crystalline form of this polypeptide or the polypeptide complexed with a ligand.

In another embodiment, the invention provides a method of determining the three dimensional structure of a crystalline polypeptide comprising the Tie-2 catalytic domain. In one embodiment, the method comprises the steps of (1) obtaining a crystal of the polypeptide comprising the catalytic domain of Tie-2; (2) obtaining x-ray diffraction data for said crystal; and (3) solving the crystal structure of said crystal. The method optionally comprises the additional step of obtaining the polypeptide, with the three dimensional structure to be determined, prior to obtaining the crystal of said peptide.

In another embodiment, the method comprises the steps of (1) obtaining a crystal of the polypeptide comprising the catalytic domain of Tie-2; (2) obtaining x-ray diffraction data for said crystal; and (3) solving the crystal structure of said crystal by using said x-ray diffraction data and the atomic coordinates for the Tie-2 catalytic domain of a second polypeptide. The method optionally comprises the additional step of obtaining the polypeptide, with the three dimensional structure to be determined, prior to obtaining the crystal of said peptide.

The invention further relates to a method for identifying a compound which inhibits the catalytic activity of Tie-2 by, for example, inhibiting the binding of natural substrates such as a tyrosyl polypeptide or protein or ATP, to the catalytic domain of Tie-2. Such a compound is referred to herein as a "Tie-2 inhibitor". The method comprises the steps of (1) using a three-dimensional structure of Tie-2 as defined by the atomic coordinates of the cataytic domain of Tie-2; (2) employing the three dimensional structure to design or select a potential inhibitor; and (3) assessing the ability of the selected compound to inhibit the catalytic activity of Tie-2 . The method can also include the step of providing the compound designed or selected in step 2, for example, by synthesizing the compound or obtaining the compound from a compound library. In addition, the method can include the step of assessing the ability of the identified compound to bind to the catalytic domain of Tie-2 and/or assessing the ability of the identified compound to inhibit the binding of a natural ligand of Tie-2.

In another embodiment, the method for identifying a compound which inhibits the catalytic activity of Tie-2, comprises the step of determining the ability of one or more functional groups and/or moieties of the compound, when present in, or bound to, the Tie-2 catalytic domain, to interact with one or more subsites of the Tie-2 catalytic domain. Generally, the Tie-2 catalytic domain is defined by the conserved homologous seqences when compared to other known tyrosine kinases. If the compound is able to interact with a preselected number or set of subsites, or has a calculated interaction energy within a desired or preselected range, the compound is identified as a potential inhibitor of Tie-2.

The invention further provides a method of designing a compound which is a potential inhibitor of the catalytic activity of Tie-2. The method includes the steps of (1) identifying one or more functional groups capable of interacting with one or more subsites of the Tie-2 catalytic domain; and (2) identifying a scaffold which presents the functional group, or functional groups, identified in step 1 in a suitable orientation for interacting with one or more subsites of the Tie-2 catalytic domain. The compound which results from attachment of the identified functional groups or moieties to the identified scaffold is a potential inhibitor of Tie-2. The Tie-2 catalytic domain is, generally, defined by the atomic coordinates of a polypeptide comprising the Tie-2 catalytic domain.

In yet another embodiment, the invention provides compounds which inhibit the catalytic activity of Tie-2 and which fit, or bind to, the Tie-2 catalytic domain. Such compounds typically comprise one or more functional groups which, when the compound is bound in the Tie-2 catalytic domain, interact with one or more subsites of the catalytic domain. Generally, the Tie-2 catalytic domain is defined by the conserved homologous sequence when compared to other known tyrosine kinases. In a particular embodiment, the Tie-2 inhibitor is a compound which is identified or designed by a method of the present invention.

The present invention further provides a method for treating a condition mediated by Tie-2 in a patient. The method comprises administering to the patient a therapeutically or prophylactically effective amount of a compound which inhibits the catalytic activity of Tie-2, such as a Tie-2 inhibitor of the invention, for example, a compound identified as a Tie-2 inhibitor or designed to inhibit Tie-2 by a method of the present invention.

The present invention provides several advantages. For example, the invention provides the first detailed three dimensional structures of the ligand binding domain of a Tie-2 protein. The methods described herein can be used to facilitate formation of Tie-2 crystals which diffract at high resolution. These structures enable the rational development of inhibitors of Tie-2 by permitting the design and/or identification of molecular structures having features which facilitate binding to the Tie-2 binding domain.

The methods of use of the structures disclosed herein, thus, permit more rapid discovery of compounds which are potentially useful for the treatment of conditions which are mediated, at least in part, by Tie-2 activity.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 presents the amino acid sequence of human Tie-2 (SEQ ID NO: 1).

Fig. 2 presents the amino acid sequence which includes the catalytic domain of human Tie-2 from amino acid 802 to amino acid 1124, and has a catalytically inactive point mutation at amino acid 964 (SEQ ID NO: 2).

Fig. 3A-3OO present the atomic coordinates for SEQ ID NO 2/inhibitor I complex.

Fig. 4A-4OO present the atomic coordinates for SEQ ID NO 2/inhibitor II complex.

Fig. 5A-5RR present the atomic coordinates for SEQ ID NO 2/inhibitor III complex.

Fig. 6A-6NN present the atomic coordinates for SEQ ID NO 2/inhibitor IV complex.

Fig. 7 shows the structure of a prototypical kinase, insulin receptor kinase.

Fig. 8 shows identifies regions of a pyrrolopyrimidine inhibitor (i.e., inhibitor I) which interact with the catalytic domain of Tie-2.

Fig. 9 shows a model of the catalytic domain of Tie-2 bound to inhibitor I. Subsites are shown in different colors.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the x-ray crystallographic study of polypeptides comprising the catalytic domain of Tie-2. The atomic coordinates which result from this study are of use in identifying compounds which fit in the catalytic domain and are, therefore, potential inhibitors of Tie-2. These Tie-2 inhibitors are of use in methods of

treating a patient having a condition which is modulated by or dependent upon Tie-2 activity, for example, a condition dependent on persistant angiogenesis.

There are at least 400 enzymes identified as protein kinases. These enzymes catalyze the phosphorylation of target protein substrates. The phosphorylation is usually a transfer reaction of a phosphate group from ATP to the protein substrate. The specific structure in the target substrate to which the phosphate is transferred is a tyrosine, serine or threonine residue. Since these amino acid residues are the target structures for the phosphoryl transfer, these protein kinase enzymes are commonly referred to as tyrosine kinases or serine/threonine kinases.

The phosphorylation reactions, and counteracting phosphatase reactions, at the tyrosine, serine and threonine residues are involved in countless cellular processes that underlie responses to diverse intracellular signals (typically mediated through cellular receptors), regulation of cellular functions, and activation or deactivation of cellular processes. A cascade of protein kinases often participate in intracellular signal transduction and are necessary for the realization of these cellular processes. Because of their ubiquity in these processes, the protein kinases can be found as an integral part of the plasma membrane or as cytoplasmic enzymes or localized in the nucleus, often as components of enzyme complexes. In many instances, these protein kinases are an essential element of enzyme and structural protein complexes that determine where and when a cellular process occurs within a cell.

Protein Tyrosine Kinases. Protein tyrosine kinases (PTKs) are enzymes which catalyse the phosphorylation of specific tyrosine residues in cellular proteins. This post-translational modification of these substrate proteins, often enzymes themselves, acts as a molecular switch regulating cell proliferation, activation or differentiation (for review, see Schlessinger and Ulrich, 1992, *Neuron* 9:383-391). Aberrant or excessive PTK activity has been observed in many disease states including benign and malignant proliferative disorders as well as diseases resulting from inappropriate activation of the immune system (e.g., autoimmune disorders), allograft rejection, and graft vs. host disease. In addition, endothelial-cell specific receptor PTKs such as KDR and Tie-2

mediate the angiogenic process, and are thus involved in supporting the progression of cancers and other diseases involving inappropriate vascularization (e.g., diabetic retinopathy, choroidal neovascularization due to age-related macular degeneration, psoriasis, rheumatoid arthritis, retinopathy of prematurity, infantile hemangiomas, psoriasis and atherosclerosis.

Tyrosine kinases can be of the receptor-type (having extracellular, transmembrane and intracellular domains) or the non-receptor type (being wholly intracellular).

Receptor Tyrosine Kinases (RTKs). The RTKs comprise a large family of transmembrane receptors with diverse biological activities. At present, at least nineteen (19) distinct RTK subfamilies have been identified. The receptor tyrosine kinase (RTK) family includes receptors that are crucial for the growth and differentiation of a variety of cell types (Yarden and Ullrich, *Ann. Rev. Biochem.* 57:433-478, 1988; Ullrich and Schlessinger, *Cell* 61:243-254, 1990). The intrinsic function of RTKs is activated upon ligand binding, which results in phosphorylation of the receptor and multiple cellular substrates, and subsequently in a variety of cellular responses (Ullrich & Schlessinger, 1990, *Cell* 61:203-212). Thus, receptor tyrosine kinase mediated signal transduction is initiated by extracellular interaction with a specific growth factor (ligand), typically followed by receptor dimerization, stimulation of the intrinsic protein tyrosine kinase activity and receptor trans-phosphorylation. Binding sites are thereby created for intracellular signal transduction molecules and lead to the formation of complexes with a spectrum of cytoplasmic signaling molecules that facilitate the appropriate cellular response. (e.g., cell division, differentiation, metabolic effects, changes in the extracellular microenvironment) see Schlessinger and Ullrich, 1992, *Neuron* 9:1-20.

Proteins with SH2 (src homology -2) or phosphotyrosine binding (PTB) domains bind activated tyrosine kinase receptors and their substrates with high affinity to propagate signals into cell. Both of the domains recognize phosphotyrosine. (Fantl *et al.*, 1992, *Cell* 69:413-423; Songyang *et al.*, 1994, *Mol. Cell. Biol.* 14:2777-2785; Songyang *et al.*, 1993, *Cell* 72:767-778; and Koch *et al.*, 1991, *Science* 252:668-678; Shoelson, *Curr. Opin. Chem. Biol.* (1997), 1(2), 227-234; Cowburn, *Curr. Opin. Struct. Biol.*

(1997), 7(6), 835-838). Several intracellular substrate proteins that associate with receptor tyrosine kinases (RTKs) have been identified. They may be divided into two principal groups: (1) substrates which have a catalytic domain; and (2) substrates which lack such a domain but serve as adapters and associate with catalytically active molecules (Songyang *et al.*, 1993, *Cell* 72:767-778). The specificity of the interactions between receptors or proteins and SH2 or PTB domains of their substrates is determined by the amino acid residues immediately surrounding the phosphorylated tyrosine residue. For example, differences in the binding affinities between SH2 domains and the amino acid sequences surrounding the phosphotyrosine residues on particular receptors correlate with the observed differences in their substrate phosphorylation profiles (Songyang *et al.*, 1993, *Cell* 72:767-778). Observations suggest that the function of each receptor tyrosine kinase is determined not only by its pattern of expression and ligand availability but also by the array of downstream signal transduction pathways that are activated by a particular receptor as well as the timing and duration of those stimuli. Thus, phosphorylation provides an important regulatory step which determines the selectivity of signaling pathways recruited by specific growth factor receptors, as well as differentiation factor receptors.

Several receptor tyrosine kinases such as FGFR-1, PDGFR, TIE-2 and c-Met, and growth factors that bind thereto, have been suggested to play a role in angiogenesis, although some may promote angiogenesis indirectly (Mustonen and Alitalo, *J. Cell Biol.* 129:895-898, 1995).

Tie-2 (TEK) is a member of a recently discovered family of endothelial cell specific receptor tyrosine kinases which is involved in critical angiogenic processes, such as vessel branching, sprouting, remodeling, maturation and stability. Tie-2 is the first mammalian receptor tyrosine kinase for which both agonist ligand(s) (e.g., Angiopoietin1 ("Ang1"), which stimulates receptor autophosphorylation and signal transduction), and antagonist ligand(s) (e.g., Angiopoietin2 ("Ang2")), have been identified. Knock-out and transgenic manipulation of the expression of Tie-2 and its ligands indicates tight spatial and temporal control of Tie-2 signaling is essential for the proper development of new

vasculature. The current model suggests that stimulation of Tie-2 kinase by the Ang1 ligand is directly involved in the branching, sprouting and outgrowth of new vessels, and recruitment and interaction of periendothelial support cells important in maintaining vessel integrity and inducing quiescence. The absence of Ang1 stimulation of Tie-2 or the inhibition of Tie-2 autophosphorylation by Ang2, which is produced at high levels at sites of vascular regression, may cause a loss in vascular structure and matrix contacts resulting in endothelial cell death, especially in the absence of growth/survival stimuli. The situation is however more complex, since at least two additional Tie-2 ligands (Ang3 and Ang4) have recently been reported, and the capacity for heterooligomerization of the various agonistic and antagonistic angiopoietins, thereby modifying their activity, has been demonstrated. Targeting Tie-2 ligand-receptor interactions as an antiangiogenic therapeutic approach is thus less favored and a kinase inhibitory strategy preferred.

The soluble extracellular domain of Tie-2 ("ExTek") can act to disrupt the establishment of tumor vasculature in a breast tumor xenograft and lung metastasis models and in tumor-cell mediated ocular neovasculatization. By adenoviral infection, the *in vivo* production of mg/ml levels ExTek in rodents may be achieved for 7-10 days with no adverse side effects. These results suggest that disruption of Tie-2 signaling pathways in normal healthy animals may be well tolerated. These Tie-2 inhibitory responses to ExTek may be a consequence of sequestration of ligand(s) and/or generation of a nonproductive heterodimer with full-length Tie-2.

Recently, significant upregulation of Tie-2 expression has been found within the vascular synovial pannus of arthritic joints of humans, consistent with a role in the inappropriate neovascularization. This finding suggests that Tie-2 plays a role in the progression of rheumatoid arthritis. Point mutations producing constitutively activated forms of Tie-2 have been identified in association with human venous malformation disorders. Tie-2 inhibitors are, therefore, useful in treating such disorders, and in other situations of inappropriate neovascularization.

The Examples herein describe the preparation and crystallization of polypeptides comprising the catalytic domain of human Tie-2. As used herein, the term "catalytic domain"

refers to a specific module common to all kinases which bind ATP, such as the tyrosyl binding site, the site where ATP binds including the metal-ion binding region, and the site where the phosphoryl transfer occurs. For Tie-2, the catalytic domain is defined by amino acid residues from about residue 828 to about residue 985 of SEQ ID NO: 1, with residues 828-840, 853-855, 872, 873, 876, 879, 880, 885-888, 900, 902-909, 912, 954, 955, 960, 964, 968-971, and 980-985 included in the catalytic domain.

The amino acid sequences of native human Tie-2 (SEQ ID NO: 1) is taken as defined in SWISS-PROT (Ziegler, et al. *Oncogene,* **8**:663 (1993)). As described in the Examples, certain of these crystals were examined by x-ray crystallography and atomic coordinates for the peptide were obtained. In certain cases, the polypeptide was unligated, that is, not complexed with a ligand. In other cases, the polypeptide was complexed with a ligand and the atomic coordinates of the ligand bound to the Tie-2 catalytic domain were also obtained.

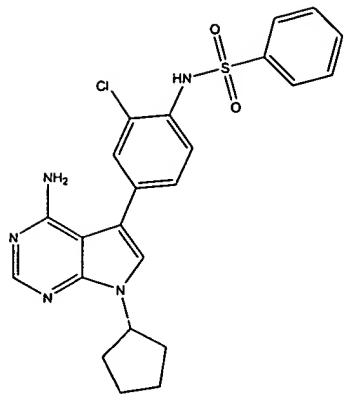
Tie-2 is subject to autophosphorylation and transphosphorylation by other proteins. Phosphorylation state is a particularly important posttranslational modification to consider. A wild-type construct (i.e., without the D964N mutation) having residues 802-1124 of SEQ ID NO 1 was isolated from an expression system as a singly- or a multiply-phosphorylated species. One singly-phosphorylated species has its phosphate on either Y897 or Y899. In multiply phosphorylated species, phosphorylation can be on combinations of many Y residues on the protein. A diphosphorylated species crystallized in the space group P2(1)2(1)2(1) with the unit cell dimensions of a = 54.320 Å, b = 75.872 Å, c = 78.143 Å, and $\alpha = \beta = \gamma = 90.0^{\circ}$. The term "space group" is a term of art which refers to the collection of symmetry elements of the unit cell of a crystal. Other phosphorylation sites are described in Jones, N., *et al., J. Biol. Chem.* (1999), *274*(43):30896.

A catalytically inactive mutant of human Tie-2 (SEQ ID NO 2) was also crystallized. The catalytically inactive mutant had the same sequence as residues 802 to 1124 of human Tie-2 except that residue 964 which is aspartic acid in wild type human Tie-2 was replaced with asparagine. This substitution rendered the mutant catalytically

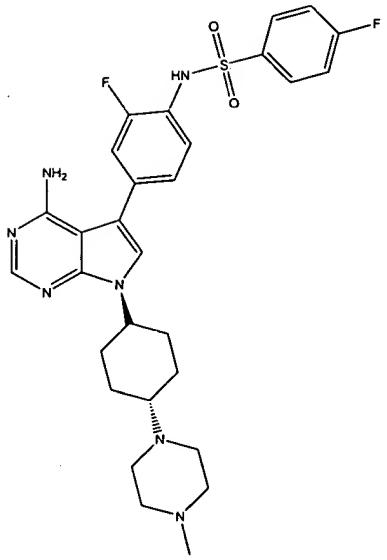
inactive. SEQ ID NO 2 crystallized in the space group C222(1) which had the unit cell dimensions a = 75.195 Å, b = 116.287 Å, c = 95.060 Å, and $\alpha = \beta = \gamma = 90.0^{o}$

The atomic coordinates for four crystals of Tie-2/ligand complexes examined by x-ray crystallography are presented in Figs. 3A-3OO, 4A-4OO, 5A-5RR and 6A-6NN. The term "atomic coordinates" (or "structural coordinates") refers to mathematical coordinates derived from mathematical equations related to the patterns obtained on diffraction of x-rays by atoms (scattering centers) of a crystalline polypeptide comprising a Tie-2 catalytic domain molecule. The diffraction data are used to calculate an electron density map of the repeating unit of the crystal. The electron density maps are used to establish the positions of the individual atoms within the unit cell of the crystal. Atomic coordinates can be transformed as is known in the art to different coordinate systems without affecting the relative positions of the atoms.

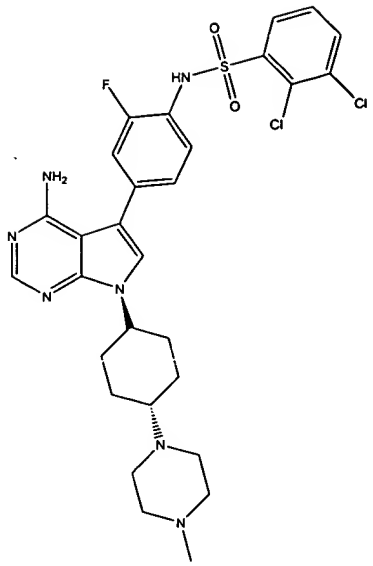
In particular, four high resolution crystal structures were obtained for SEQ ID NO 2) complexed with one of four different inhibitors shown below:



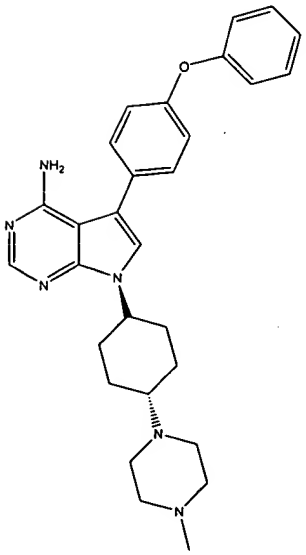
Inhibitor I



Inhibitor II



Inhibitor III



Inhibitor IV

The results of the x-ray crystal structure determination for SEQ ID NO 2, the catalytic domain of human Tie-2, showed the following features:

The overall structure adopted a recognizable kinase fold with an N-terminal lobe and a somewhat larger C-terminal lobe. ATP binding was at the interface of the two lobes with inhibitors also binding in this region. The major secondary structural elements of the N-terminal lobe were a five strand beta sheet and a long alpha helix. The C-terminal lobe was primarily a bundle of alpha helices with a short, two-strand beta sheet near the interface with the N-terminal lobe. Fig. 7 shows a prototypical receptor tyrosine kinase, insulin receptor kinase which highlights the structural features associated with known kinases. The structure of the catalytic domain of Tie-2, shown in Fig. 9 has similar features to this

The hinge region connects the N-terminal and C-terminal lobes. The portion of the hinge which forms part of the ATP/inhibitor binding region presents several hydrogen bonding partners. The carbonyl oxygen atoms of E903, A905 and P906 and the backbone amide protons of A905, H907 and G908 were presented into the pocket. The sidechains of L900, I902, Y904 and A905 helped to define the size, shape and nature of the binding pocket.

The purine core binding region was the region where the N-terminal and C-terminal lobes of the protein cooperate to form a flat, predominantly hydrophobic binding region which is the traditional location for the purine ring of ATP in other kinase structures. The residues which contribute to this region included: I830, A853, V838, I886, L971 and A981. Sidechains of residues in the hinge region, I902, Y904 and A905 also contributed hydrophobic character to this region. The carbonyl oxygen of I830 and the amide proton of V838 also presented an interaction site within this region.

By analogy to known kinase structures, the ribose ring of ATP would traditionally occupy an area between G831 in the N-terminal lobe and N909 in the C-terminal lobe called the extended sugar pocket. The backbone amide protons of G831, E832 and N909, the carbonyl oxygen of R968 and the sidechains of E832, N909 and D912 presented hydrogen bond partners.

By anaology to known kinase structures, the γ-phosphate of ATP would occupy an area around the sidechains of residues D964 (N964 in the catalytically inactive mutant,

SEQ ID NO 2). The sidechain of R968 also contributes to this region. The predominant available interaction type was hydrogen bonding, with quite complex coordination possible.

The nucleotide binding loop, or glycine-rich loop, was a flap like loop in the N-terminal lobe which covered the front portion of the ATP binding region. Residues not already described in other binding areas include D828, V829, G833, N834, F835, G836, Q837, L839, and K840. Residues I830, G831, E832 and V838 were also part of this structural element, but have already been included in other binding regions described above. This loop is usually considered to be very flexible and is capable of altering the shape and size of the ATP binding region. Carbonyl oxygen atoms, N834 sidechain atoms and backbone amide protons of G833, N834 and F835 were potentially available for hydrogen bonding. The sidechain atoms of D828 and K840 were available for ionic/hydrogen bonding interactions. The sidechain atoms of V829, I830, F835 and L839 can contribute to hydrophobic interactions.

The early activation loop was a long flexible loop containing at least one residue, the phosphorylation of which, is generally believed to determine the activation state of the protein. The loop begins in the ATP binding site and ends in the C-terminal lobe in the area which most likely corresponds to substrate binding. Residues F983, G984, and L985 form part of the ATP binding site and were also on the N-terminal side of the activation loop. The carboxyl oxygen and amide protons of F983 and G984 and the amide proton of L985 were available for hydrogen bonding and the sidechains of F983 and L985 can contribute to hydrophobic interactions.

K855, by homology to known kinases, is part of the catalytic mechanism of the kinase. The amino group can participate in ionic or hydrogen bond interactions and the methylene groups can contribute to hydrophobic interactions. The sidechain is very mobile.

The distal hydrophobic pocket is is characterized by a buried hydrophobic cavity. This portion of the ATP binding region is not occupied by any ATP atoms in known kinase structures. Residues which contribute to this pocket include L873, L876, L879,

I885, L888, Y954, L955, F960 and I980. I886, A981 and F983 from regions already described also contribute hydrophobic interactions to this region. In addition, there was a number of backbone hydrogen bond partners available in this area. These partners included the carbonyl oxygen atoms of I886, L879, and G880. With the apparent disruption of the alpha-C helix, carbonyl oxygen atoms of E872, L873 and L876 may also be available. The backbone amide proton of residues, I886 and L888 were also available in this region.

Several residues contributed to the ATP/inhibitor binding site but do not seem to be part of definable subregion. These residues are I854, E872, N887, I970 and I980. E872 often forms an ionic interaction with the catalytic lysine in known kinase structures. N887 may contribute to the distal hydrophobic pocket. The sidechains of I854, and I970 face away from the ATP pocket, however carbonyl oxygen atoms from these residues as well as I980 were presented towards the binding region.

The structure of the SEQ ID NO 2/inhibitor I complex had the following features:

Final resolution of the structure was 2.8 Å in space group C2221, with final coordinates determined for backbone atoms of residues 818-857, 864-995, 1001-1116.

The pyrrolopyrimidine ring of inhibitor I formed hydrogen bonds to residues in the hinge region and interacts with purine core region. The core of the inhibitor presented a hydrogen bond donor in the form of the amino proton of the 4-NH_2 substituent to the carbonyl oxygen of E903. Atom N3 of the pyrimidine ring accepted a hydrogen bond from the backbone N-H of A905. The ring system of the core presented a planar face to residues of both the C-terminal and N-terminal lobes. The residues in these areas present a hydrophobic surface which "sandwiches" the planar core of the inhibitor. Residues involved in this hydrophobic sandwich region include I830, V838, I86, I902 and L971. Atoms N1 and N7 of the inhibitor core faced the solvent exposed mouth of the binding pocket. Atom C6 of the inhibitor faced the long axis of the nucleotide binding loop of the N-terminal lobe of the protein.

The N7 cyclopentane ring of Inhibitor I was directed towards solvent but was still within the protein cavity. This region was described above as the extended sugar pocket.

This region was characterized by hydrophobic interactions with primarily I830 and L971. Methylene groups of E832 may also contribute in this fashion.

The phenyl ring attached to C5 of the pyrrolopyrimidine ring was in a predominantly hydrophobic area, generated by residues from the purine core region, the distal hydrophobic pocket and methylene groups from the catalytic lysine, K855. The hydrophobic contacts were with residues V838, I886, I902, L971 and A981. Lysine 855 was highly mobile, so it is also possible that the chlorine atom meta to the pyrrolopyrimidine ring was receiving a hydrogen bond.

The sulfonamide linker made a clear hydrogen bond with an amide proton of D982 and may also make a hydrogen bond to the amide proton of F983.

The terminal phenyl ring was located in the distal hydrophobic pocket. Primary contacts were with L876, I886, L888 and F983.

The structure of the SEQ ID NO 2/inhibitor II, SEQ ID NO 2/inhibitor III and SEQ ID NO 2/inhibitor IV complexes had the following features:

Residues 818-857, 864-995, 1001-1116 have been modeled into the solved structure. A space group P42212 was observed. The overall fold is still a standard kinase catalytic domain fold and the binding regions described above for SEQ ID NO 2/inhibitor I still pertain.

The pyrrolopyrimidine core, B-ring, linker and C-ring of inhibitor II in the SEQ ID NO 2/inhibitor II complex was bound the same way as inhibitor I. In addition, the N-7 cyclohexyl N-methyl piperazinyl group occupied the extended sugar pocket and made a strong ionic interaction with D912.

The pyrrolopyrimidine of inhibitor III binds was bound the same way in the SEQ ID NO 2/inhibitor III complex as inhibitor I. The N-7 cyclohexyl N-methy piperazinyl group occupied the extended sugar pocket and made a strong ionic interaction with D912 as in the SEQ ID NO 2/inhibitor II complex. The B-ring was bound in a similar fashion to inhibitor I, however, the hydrogen bond between halogens, fluorine in this case, and K855 was more clear. The sulfonyl oxygens of the sulfonamide linker made two clear hydrogen bonds to backbone amide protons of D983 and F983. The C-ring occupied the

distal hydrophobic pocket with main interactions coming from L876, I886, L888, L900, I902 and F983.

The pyrrolopyrimidine core of inhibitor IV in the SEQ ID NO 2/inhibitor IV complex was bound the same way as inhibitor I. The N-7 cyclohexyl N-methyl piperazinyl group occupies the extended sugar pocket and makes a strong ionic interaction with D912 as in SEQ ID NO 2/inhibitor II. The B-ring binds in a similar fashion to inhibitor I, however there is no halogen atom to act as a potential hydrogen bond partner in inhibitor IV. The oxygen atom of the linker accepted a hydrogen bond from the catalytic lysine, K855. The C-ring of inhibitor IV occupied the distal hydrophobic pocket with main interactions coming from L876, I886, I902 and F983.

Analysis of the three dimensional structure of the Tie-2 catalytic domain has indicated the presence of a number of subsites, each of which includes molecular functional groups capable of interacting with complementary moieties of an inhibitor. Subsites 1 through 9 of the Tie-2 catalytic domain are defined below. A summary of the properties of the chemical moieties present at each subsite is given below. Subsites are characterized below according to the properties of chemical moieties with which they are complementary, or with which they can interact. Such moieties can include hydrogen bond acceptors, such as hydroxyl, amino, ether, thioether, carboxyl, P=O, and carbonyl groups, halogen atoms, such as fluorine, chlorine, bromine and iodine atoms; and other groups including a heteroatom having at least one lone pair of electrons, such as groups containing trivalent phosphorous, di- and tetravalent sulfur, oxygen and nitrogen atoms; hydrogen bond donors, such as hydroxyl, thiol, an amide proton, amine protons, carboxylic acid groups and any of the groups listed under hydrogen atom acceptors to which a hydrogen atom is bonded; hydrophobic groups, such as linear, branched or cyclic alkyl, ether or thioalkyl groups; linear, branched or cyclic alkenyl groups; linear, branched or cyclic alkynyl groups; aryl groups, such as mono- and polycyclic aromatic hydrocarbyl groups and mono- and polycyclic heterocyclic or heteroaryl groups; positively charged groups, such as primary, secondary, tertiary and quaternary ammonium groups, imidazolium and other protonated heteroalkyl and heteroaryl moieties, substituted and

unsubstituted guanidinium groups, sulfonium groups and phosphonium groups; and negatively charged groups, such as carboxylate, phenolate, thiolate, sulfonamide, sulfamate, boronate, vanadate, sulfonate, sulfinate, phosphinate, tetrazolate and other heteroaryl anions, heterocyclic N-oxides, and phosphonate groups. A given chemical moiety can contain one or more of these groups.

Subsite 1: Hinge Region

Hydrogen Acceptors: The the backbone carbonyl oxygen of residues E903, A905 and P906 present proton acceptors.

Hydrogen Donors: The backbone amide protons of residues A905, H907 and G908 present proton donors.

Hydrophobic Groups: The sidechains of L900, I902, Y904 and A905 present hydrophobic groups.

Subsite 2: The Purine Core Binding Region

Hydrophobic Groups: Residues I830, A853, V838, I886, L971, A981 and the sidechains of residues I902, Y904, and A905 present hydrophobic groups.

Hydrogen Acceptors: The carbonyl oxygen of I830 presents a proton acceptor.

Hydrogen Donors: The amide proton of V838 presents a proton donor.

Subsite 3: The Extended Sugar Pocket

Hydrogen Acceptors: The backbone carbonyl oxygen of R968 and the sidechain carbonyl oxygen of E832, N909 and D912 present proton acceptors.

Hydrogen Donors: The backbone amide protons of G831, E832 and N909 present proton donors.

Subsite 4: The Extended γ-Phosphate Region

Hydrogen Bonding Groups: Residues D964 (N964 in the catalytically inactive mutant), N969 and D982 present both proton donor and proton acceptor groups.

Subsite 5: The Nucleotide binding Loop

Hydrogen Acceptors: The carbonyl oxygen of the sidechain of residue N834 presents a proton acceptor.

Hydrogen Donors: The backbone amide protons of residues G833, N834 and F835 present proton donors.

Positively Charged Group: The sidechain of K840 presents a positively charged site.

Negatively Charged Group: The sidechain of D828 presents a negatively charged site.

Hydrophobic Groups: The sidechains of V829, I830, F835 and L839 present hydrophobic groups.

Subsite 6: The Early Activation Loop

Hydrogen Acceptors: The backbone carbonyl oxygens of residues F983 and G984 presents a proton acceptor.

Hydrogen Donors: The backbone amide protons of residues F983, G984 and L985 present proton donors.

Hydrophobic Groups: The sidechains of F983 and L985 present hydrophobic groups.

Subsite 7: The Catalytic Lysine

Positively Charged Group: The sidechain of K855 presents a positively charged site.

Hydrophobic Group: The methylene groups of the sidechain of K855 presents a hydrophobic group.

Subsite 8: The Distal Hydrophobic Pocket

Hydrophobic Groups: Residues L873, L876, L879, I885, L888, Y954, L955, F960, I980, I886, A981 and F983 present hydrophobic groups.

Hydrogen Acceptors: The backbone carbonyl oxygens of residues I886, L879, G880, E872, L873 and L876 present proton acceptors.

Hydrogen Donors: The backbone amide protons of residues I886 and L888 present proton donors.

Subsite 9: Miscellaneous interaction sites which contribute to the ATP binding site. Hydrogen Acceptors: The backbone carbonyl oxygens of residues I854, I970 and I980 present proton acceptors in the ATP binding region.
Negatively Charged Groups: E872 presents a negatively charged group which often forms an ionic bond with the catalytic lysine residue K855.

Fig. 9 provides a model of the catalytic domain of Tie-2 bound to inhibitor I. Subsites 1-9 of the catalytic domain are each depicted in a different color as follows: the hinge region (dark blue), the purine core (light blue), the extended sugar pocket (light purple), the γ-phosphate region (dark yellow), the nucleotide binding loop (red), the early activation loop (dark green), the catalytic lysine (light green), the distal hydrophobic pocket (dark purple), and miscellaneous interaction sites (brown). The inhibitor is depicted in light yellow.

In one embodiment, the present invention provides polypeptides comprising the catalytic domain of Tie-2, crystalline forms of these polypeptides, optionally complexed with a ligand, and the three dimensional structure of the polypeptides, including the three dimensional structure of the Tie-2 catalytic domain. In general, these three dimensional structures are defined by atomic coordinates derived from x-ray crystallographic studies of the polypeptides. The catalytic domain can be unphosphorylated, monophosphorylated or multiply phosphorylated. Phosphorylization typically occurs at tyrosine residues. One monophosphorylated species has a phosphate group on Y897 or Y899.

The polypeptides can include the catalytic domain of Tie-2 from any species, such as a yeast or other unicellular organism, an invertebrate or a vertebrate. Preferably, the polypeptide includes the catalytic domain of a mammalian Tie-2, such as murine Tie-2. More preferably, the polypeptide includes the catalytic domain of human Tie-2. The polypeptides of the invention also includes polypeptides comprising single nucleotide

polymorphisms of the catalytic domain of human Tie-2 or murine Tie-2. In one embodiment, the polypeptides of the invention, and crystalline forms thereof, include a sequence which has at least 80% identity to the catalytic domain of human Tie-2 or murine Tie-2.

To determine the percent identity of two amino acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment, and non-homologous (dissimilar) sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a first sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the second sequence. The amino acid residues at corresponding amino acid positions are then compared. When a position in the first sequence is occupied by the same amino acid residue as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The invention also encompasses polypeptides having a lower degree of identity but having sufficient homology so as to perform one or more of the same functions performed by Tie-2 polypeptides described herein by amino acid sequence. Homology for a polypeptide is determined by conservative amino acid substitution. Such substitutions are those that substitute a given amino acid in a polypeptide by another amino acid of like characteristics. Conservative substitutions are likely to be phenotypically silent. Typically seen as conservative substitutions are the replacements, one for another, for example, among the aliphatic amino acids Ala, Val, Leu, and Ile; interchange of the hydroxyl residues Ser and Thr, exchange of the acidic residues Asp and Glu, substitution between the amide residues Asn and Gln, exchange of the basic residues Lys and Arg or replacements among the aromatic residues Phe, Tyr and Trp. Guidance

concerning which amino acid changes are likely to be phenotypically silent is found in Bowie *et al., Science 247*:1306-1310 (1990).

The comparison of sequences and determination of percent identity and homology between two sequences can be accomplished using a mathematical algorithm. (*Computational Molecular Biology*, Lesk, A.M.,ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data, Part 1,* Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology,* von Heinje, G., Academic Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereaux, J., eds., M. Stockton Press, New York, 1991). In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (*J. Mol. Biol.* (48):444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available on March 29, 2000 at http://www.gcg.com), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of, for example, 16, 14, 12, 10, 8, 6, or 4 and a length weight of, for example, 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (Devereux, J., *et al., Nucleic Acids Res. 12(1)*:387 (1984)) (available on March 29, 2000 at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of, for example, 40, 50, 60, 70, or 80 and a length weight of, for example, 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (*CABIOS, 4*:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using, for example, a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The protein sequences of the present invention, for example, amino acids 802-1124 of human Tie-2 (SEQ ID NO 1), can further be used as a "query sequence" to perform a search against databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST

programs (version 2.0) of Altschul, *et al.* (*J. Mol. Biol. 215*:403-10 (1990)). BLAST protein searches can be performed with the XBLAST program, for example, score = 50, word length = 3, to obtain amino acid sequences homologous to the proteins of the invention. To obtain gapped alignments for comparison purposes, gapped BLAST can be utilized as described in Altschul *et al., (Nucleic Acids Res. 25*(17):3389-3402 (1997)). When utilizing BLAST and gapped BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used as given on March 29, 2000 at http://www.ncbi.nlm.nih.gov.

Homology for amino acid sequences can be defined in terms of the parameters set by the Advanced Blast search available from NCBI (the National Center for Biotechnology Information; see, for Advanced BLAST , www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-newblast?Jform=1 (on March 29, 2000)). These default parameters, recommended for a query molecule of length greater than 85 amino acid residues or nucleotides have been set as follows: gap existence cost, 11, per residue gap cost, 1; lambda ratio, 0.85. Further explanation of version 2.0 of BLAST can be found on related website pages and in Altschul, S.F. *et al., Nucleic Acids Res. 25*:3389-3402 (1997).

In one embodiment, the polypeptide includes amino acids 802 to 1124 of SEQ ID NO: 1. Polypeptides can also have amino acids 792 to 1124, 782 to 1124, 772 to 1124, 812 to 1124, 822 to 1124, 832 to 1124, 802 to 1114, 802 to 1104, or 802 to 1094 of SEQ ID NO 1. In another embodiment, the polypeptide can be a catalytically inactive mutant of Tie-2, such as SEQ ID NO 2, wherein the asparagine amino acid at 964 is replaced with an aspartic acid amino acid (designated D964N mutant). Other catalytically inactive mutants include substitution of the asparagine amino acid at 964 with alanine, serine, threonine, or glycine.

In another embodiment, the catalytic domain which is crystallized can have deletions of amino acids from the native sequence. For example, a polypeptide which is suitable for crystallization can include amino acids 802 to 918 of SEQ ID NO 1 fused to amino acids 934 to 1124 of SEQ ID NO 1 or other related "kinase-insert domain" deletions.

The crystalline polypeptide, preferably, further includes a ligand bound to the Tie-2 catalytic domain. The ligand is, preferably, a small (less than about 1500 molecular weight) organic molecule, for example, inhibitor I, II, III, or IV.

In one embodiment, the invention relates to a method of determining the three dimensional structure of a first polypeptide comprising the catalytic domain of a Tie-2 protein. The method includes the steps of (1) obtaining a crystal comprising the first polypeptide; (2) obtaining x-ray diffraction data for said crystal; and (3) using the x-ray diffraction data and the atomic coordinates of a second polypeptide comprising the catalytic domain of a Tie-2 protein to solve the crystal structure of the first polypeptide, thereby determining the three dimensional structure of the first polypeptide. The second polypeptide can include the same Tie-2 catalytic domain as the first polypeptide, or a different Tie-2 catalytic domain. Either or both of the first and second polypeptides can, optionally, be complexed with a ligand. That is, the crystal of the first polypeptide can comprise a complex of the first polypeptide with a ligand. The atomic coordinates of the second polypeptide can, optionally, include the atomic coordinates of a ligand molecule bound to the second polypeptide. The atomic coordinates of the second polypeptide, generally, have been previously obtained, for example, by x-ray crystallographic analysis of a crystal comprising the second polypeptide or a complex of the second polypeptide with a ligand. The atomic coordinates of the second polypeptide can be used to solve the crystal structure using methods known in the art, for example, molecular replacement or isomorphous replacement. Preferably, the second polypeptide comprises the catalytic domain of a mammalin Tie-2, more preferably, human Tie-2. For example the atomic coordinates which can be used include the atomic coordinates presented herein, preferably the atomic coordinates presented in Figures 3-7.

The invention also provides a method of identifying a compound which is a potential inhibitor of Tie-2. The method comprises the steps of (1) obtaining a crystal of a polypeptide comprising the catalytic domain of Tie-2; (2) obtaining the atomic coordinates of the polypeptide by x-ray diffraction studies using said crystal; (3) using said atomic coordinates to define the catalytic domain of Tie-2; and (4) identifying a

compound which fits the catalytic domain. The method can further include the steps of obtaining, for example, from a compound library, or synthesizing the compound identified in step 4, and assessing the ability of the identified compound to inhibit Tie-2 enzymatic activity.

The polypeptide preferably comprises the catalytic domain of a mammalian Tie-2. More preferably the polypeptide comprises the catalytic domain of human Tie-2. In a preferred embodiment, the polypeptide is a polypeptide of the present invention, as described above.

The polypeptide can be crystallized using methods known in the art, such as the methods described in the Examples, to afford polypeptide crystals which are suitable for x-ray diffraction studies. A crystalline polypeptide/ligand complex can be produced by soaking the resulting crystalline polypeptide in a solution including the ligand. Preferably, the ligand solution is in a solvent in which the polypeptide is insoluble.

The atomic coordinates of the polypeptide (and ligand) can be determined, for example, by x-ray crystallography using methods known in the art. The data obtained from the crystallography can be used to generate atomic coordinates, for example, of the atoms of the polypeptide and ligand, if present. As is known in the art, solution and refinement of the x-ray crystal structure can result in the determination of coordinates for some or all of the non-hydrogen atoms. The atomic coordinates can be used, as is known in the art, to generate a three-dimensional structure of the Tie-2 catalytic domain. This structure can then be used to assess the ability of any given compound, preferably using computer-based methods, to fit into the catalytic domain.

A compound fits into the catalytic domain if it is of a suitable size and shape to physically reside in the catalytic domain, that is, if it has a shape which is complementary to the catalytic domain and can reside in the catalytic domain without significant unfavorable steric or van der Waals interactions. Preferably, the compound includes one or more functional groups and/or moieties which interact with one or more subsites within the catalytic domain. Computational methods for evaluating the ability of a

compound to fit into the catalytic domain, as defined by the atomic coordinates of the polypeptide, are known in the art, and representative examples are provided below.

In another embodiment, the method of identifying a potential inhibitor of Tie-2 comprises the step of determining the ability of one or more functional groups and/or moieties of the compound, when present in the Tie-2 catalytic domain, to interact with one or more subsites of the Tie-2 catalytic domain. Preferably, the Tie-2 catalytic domain is defined by the atomic coordinates of a polypeptide comprising the Tie-2 catalytic domain. If the compound is able to interact with a preselected number or set of subsites, the compound is identified as a potential inhibitor of Tie-2.

A functional group or moiety of the compound is said to "interact" with a subsite of the Tie-2 catalytic domain if it participates in an energetically favorable, or stabilizing, interaction with one or more complementary moieties within the subsite. Two chemical moieties are "complementary" if they are capable, when suitably positioned, of participating in an attractive, or stabilizing, interaction, such as an electrostatic or van der Waals interaction. Typically, the attractive interaction is an ion-ion (or salt bridge), ion-dipole, dipole-dipole, hydrogen bond, pi-pi or hydrophobic interaction. For example, a negatively charged moiety and a positively charged moiety are complementary because, if suitably positioned, they can form a salt bridge. Likewise, a hydrogen bond donor and a hydrogen bond acceptor are complementary if suitably positioned.

Typically, an assessment of interactions between the test compound and the Tie-2 catalytic domain may employ computer-based computational methods, such as those known in the art, in which possible interactions of a compound with the protein, as defined by atomic coordinates, are evaluated with respect to interaction strength by calculating the interaction energy upon binding the compound to the protein. Compounds which have calculated interaction energies within a preselected range or which otherwise, in the opinion of the computational chemist employing the method, have the greatest potential as Tie-2 inhibitors, can then be provided, for example, from a compound library or via synthesis, and assayed for the ability to inhibit Tie-2. The interaction energy for a

given compound generally depends upon the ability of the compound to interact with one or more subsites within the protein catalytic domain.

In one embodiment, the atomic coordinates used in the method are the atomic coordinates set forth in Figs. 3A-3OO, 4A-4OO, 5A-5RR and 6A-6NN. It is to be understood that the coordinates set forth in Figs. 3-6 can be transformed, for example, into a different coordinate system, in ways known to those skilled in the art without substantially changing the three dimensional structure represented thereby.

In certain cases, a moiety of the compound can interact with a subsite via two or more individual interactions. A moiety of the compound and a subsite can interact if they have complementary properties and are positioned in sufficient proximity and in a suitable orientation for a stabilizing interaction to occur. The possible range of distances for the moiety of the compound and the subsite depends upon the distance dependence of the interaction, as is known in the art. For example, a hydrogen bond typically occurs when a hydrogen bond donor atom, which bears a hydrogen atom, and a hydrogen bond acceptor atom are separated by about 2.5 Å and about 3.5 Å. Hydrogen bonds are well known in the art (Pimentel *et al.*, *The Hydrogen Bond*, San Francisco: Freeman (1960)). Generally, the overall interaction, or binding, between the compound and the Tie-2 catalytic domain will depend upon the number and strength of these individual interactions.

The ability of a test compound to interact with one or more subsites of the catalytic domain of Tie-2 can be determined by computationally evaluating interactions between functional groups, or moieties, of the test compound and one or more amino acid side chains in a particular protein subsite, such as subsites 1 to 9 above. Typically, a compound which is capable of participating in stabilizing interactions with a preselected number of subsites, preferably without simultaneously participating in significant destabilizing interactions, is identified as a potential inhibitor of Tie-2. Such a compound will interact with one or more subsites, preferably with two or more subsites and, more preferably, with three or more subsites.

The invention further provides a method of designing a compound which is a potential inhibitor of Tie-2. The method includes the steps of (1) identifying one or more functional groups capable of interacting with one or more subsites of the Tie-2 catalytic domain; and (2) identifying a scaffold which presents the functional group or functional groups identified in step 1 in a suitable orientation for interacting with one or more subsites of the Tie-2 catalytic domain. The compound which results from attachment of the identified functional groups or moieties to the identified scaffold is a potential inhibitor of Tie-2. The Tie-2 catalytic domain is, generally, defined by the conserved homolohous sequence when compared to other known tyrosine kinases, for example, the atomic coordinates set forth in Figs. 3A-3OO, 4A-4OO, 5A-5RR and 6A-6NN.

Suitable methods, as are known in the art, can be used to identify chemical moieties, fragments or functional groups which are capable of interacting favorably with a particular subsite or set of subsites. These methods include, but are not limited to: interactive molecular graphics; molecular mechanics; conformational analysis; energy evaluation; docking; database searching; pharmacophore modeling; de novo design and property estimation. These methods can also be employed to assemble chemical moieties, fragments or functional groups into a single inhibitor molecule. These same methods can also be used to determine whether a given chemical moiety, fragment or functional group is able to interact favorably with a particular subsite or set of subsites.

In one embodiment, the design of potential human Tie-2 inhibitors begins from the general perspective of three-dimensional shape and electrostatic complementarity for the catalytic domain, encompassing subsites 1-9, and subsequently, interactive molecular modeling techniques can be applied by one skilled in the art to visually inspect the quality of the fit of a candidate inhibitor modeled into the binding site. Suitable visualization programs include INSIGHTII (Molecular Simulations Inc., San Diego, CA), QUANTA (Molecular Simulations Inc., San Diego, CA), SYBYL (Tripos Inc., St Louis, MO), RASMOL (Roger Sayle *et al.*, *Trends Biochem. Sci.* **20**: 374-376 (1995)), GRASP (Nicholls *et al.*, *Proteins* **11**: 281-289 (1991)), and MIDAS (Ferrin *et al.*, *J. Mol. Graphics* **6**:13-27 (1988)).

A further embodiment of the present invention utilizes a database searching program which is capable of scanning a database of small molecules of known three-dimensional structure for candidates which fit into the target protein site. Suitable software programs include CATALYST (Molecular Simulations Inc., San Diego, CA), UNITY (Tripos Inc., St Louis, MO), FLEXX (Rarey et al., *J. Mol. Biol.* **261**: 470-489 (1996)), CHEM-3DBS (Oxford Molecular Group, Oxford, UK), DOCK (Kuntz *et al.*, *J. Mol. Biol* **161**: 269-288 (1982)), and MACCS-3D (MDL Information Systems Inc., San Leandro, CA). It is not expected that the molecules found in the search will necessarily be leads themselves, since a complete evaluation of all interactions will necessarily be made during the initial search. Rather, it is anticipated that such candidates might act as the framework for further design, providing molecular skeletons to which appropriate atomic replacements can be made. Of course, the chemical complimentary of these molecules can be evaluated, but it is expected that the scaffold, functional groups, linkers and/or monomers may be changed to maximize the electrostatic, hydrogen bonding, and hydrophobic interactions with the enzyme. Goodford (Goodford *J Med Chem* **28**:849-857 (1985)) has produced a computer program, GRID, which seeks to determine regions of high affinity for different chemical groups (termed probes) on the molecular surface of the binding site. GRID hence provides a tool for suggesting modifications to known ligands that might enhance binding.

A range of factors, including electrostatic interactions, hydrogen bonding, hydrophobic interactions, desolvation effects, conformational strain or mobility, chelation and cooperative interaction and motions of ligand and enzyme, all influence the binding effect and should be taken into account in attempts to design bioactive inhibitors.

Yet another embodiment of a computer-assisted molecular design method for identifying inhibitors comprises searching for fragments which fit into a binding region subsite and link to a predefined scaffold. The scaffold itself may be identified in such a manner. Programs suitable for the searching of such functional groups and monomers include LUDI (Boehm, *J Comp. Aid. Mol. Des.* **6**:61-78 (1992)), CAVEAT (Bartlett *et al.* in "Molecular Recognition in Chemical and Biological Problems", special publication of

The Royal Chem. Soc., **78**:182-196 (1989)) and MCSS (Miranker *et al. Proteins* **11**: 29-34 (1991)).

Yet another embodiment of a computer-assisted molecular design method for identifying inhibitors of the subject phosphatase comprises the *de novo* synthesis of potential inhibitors by algorithmic connection of small molecular fragments that will exhibit the desired structural and electrostatic complementarity with the active site of the enzyme. The methodology employs a large template set of small molecules with are iteratively pieced together in a model of the Tie-2 active site. Programs suitable for this task include GROW (Moon *et al. Proteins* **11**:314-328 (1991)) and SPROUT (Gillet *et al. J Comp. Aid. Mol. Des.* **7**:127 (1993)).

In yet another embodiment, the suitability of inhibitor candidates can be determined using an empirical scoring function, which can rank the binding affinities for a set of inhibitors. For an example of such a method see Muegge *et al.* and references therein (Muegge *et al.*, *J Med. Chem.* **42**:791-804 (1999)).

Other modeling techniques can be used in accordance with this invention, for example, those described by Cohen *et al.* (*J. Med. Chem.* **33**: 883-894 (1994)); Navia *et al.* (*Current Opinions in Structural Biology* **2**: 202-210 (1992)); Baldwin *et al.* (*J. Med. Chem.* **32**: 2510-2513 (1989)); Appelt *et al.* (*J. Med. Chem.* **34**: 1925-1934 (1991)); and Ealick *et al.* (*Proc. Nat. Acad. Sci. USA* **88**: 11540-11544 (1991)).

A compound which is identified by one of the foregoing methods as a potential inhibitor of Tie-2 can then be obtained, for example, by synthesis or from a compound library, and assessed for the ability to inhibit Tie-2 *in vitro*. Such an *in vitro* assay can be performed as is known in the art, for example, by contacting Tie-2 in solution with the test compound in the presence of a substrate for Tie-2. The rate of substrate transformation can be determined in the presence of the test compound and compared with the rate in the absence of the test compound. Suitable assays for Tie-2 biological activity are described in Example 4.

An inhibitor identified or designed by a method of the present invention can be a competitive inhibitor, an uncompetitive inhibitor or a noncompetitive inhibitor. A

"competitive" inhibitor is one that inhibits Tie-2 activity by binding fully or partially within the same region of Tie-2, as ATP, thereby directly competing with ATP for the active site of Tie-2. An "uncompetitive" inhibitor inhibits Tie-2 by binding to a different region of the enzyme than ATP. Such inhibitors bind to Tie-2 already bound with ATP and not to the free enzyme. A "non-competitive" inhibitor is one that can bind to either the free or ATP bound form of Tie-2. In some instances, an inhibitor may inhibit the enzymes catalytic activity by impeding the binding of multiple substrates (e.g., ATP and tyrosyl substrates). this may be accomplished by fully or partially occluding multiple substrate binding sites, or by occupying a site which allosterically or conformationally reduces affinities for substrates or blocks product release.

In another embodiment, the present invention provides Tie-2 inhibitors, and methods of use thereof, which are capable of binding to the catalytic domain of Tie-2, for example, compounds which are identified as inhibitors of at least one biological activity of Tie-2 or which are designed by the methods described above to inhibit at least one biological activity of Tie-2. For example, the invention includes compounds which interact with one or more, preferably two or more, and more preferably, three or more of Tie-2 subsites 1 to 9.

In one embodiment, the Tie-2 inhibitor of the invention comprises a moiety or moieties positioned to interact with subsite 1, subsite 2 and, optionally, with at least one other subsite, when present in the Tie-2 catalytic domain. For example, a functional group which can interact with subsite 1 can be a hydrogen bond donor, a hydrogen bond acceptor, or a hydrophobic moiety. A functional group which can interact with subsite 2 can be a hydrophobic group, hydrogen bond donor, or a hydrogen bond acceptor.

In another embodiment, the Tie-2 inhibitor of the invention comprises functional groups positioned to interact with subsites 1, 2 and 3, and, optionally, one or more additional subsites.

The Tie-2 inhibitors of the invention also include compounds having functional groups positioned to interact with subsite 1, subsite 2, subsite 8 and, optionally, one or more additional subsites. In another embodiment, the inhibitor has functional groups

positioned to interact with subsite 1, subsite 2, subsite 3, subsite 8, and, optionally, one or more additional subsites.

In other embodiments, the Tie-2 inhibitors of the invention include compounds which have functional groups positioned to interact with the following groups of subsites, each of which can, optionally, include one or more additional subsites:
subsites 1, 4, and 5; subsites 1, 2, 7 and 8; subsites 1, 2, 3, 7 and 8; subsites 1, 2, 3, 7 and 8; subsites 1, 2, 4, 6 and 8; subsites 1, 2, 3, 4, 6 and 8; subsites 1, 2, 3, 4, 6 and 8.

A moiety of the inhibitor compound is "positioned to interact" with a given subsite, if, when placed within the Tie-2 catalytic domain, as defined by the atomic coordinates presented in Figs. 3-6, the moiety is proximal to, and oriented properly relative to, the appropriate amino acid side chains within the subsite.

As indicated in the description of the subsites above, several of subsites 1-9 can potentially interact with two or more types of moieties. For each of the subsites listed below the preferred type of interacting moiety possessed by the potential inhibitor is indicated.

Subsite 1: hydrogen bond donor (E903) and hydrogen bond acceptor (A905).

Subsite 2: hydrophobic, preferably aromatic, moiety (I830, V838, I886, I902 and L971).

Subsite 3: hydrophobic, preferably alkyl, moiety (I830 and L971) and a positively charged moiety (D912).

Subsite 4: hydrogen acceptor moiety (D982 and F938).

Subsite 8: hydrophobic, preferably aromatic, moiety (L876, I886, L888 and F983)

A preferred Tie-2 inhibitor of the invention inhibits Tie-2 enzymatic activity with a Ki of at least about 1 mM, preferably at least about 100 μM and more preferably at least about 10 μM. In another embodiment, a Tie-2 inhibitor binds selectively to a Tie-2 receptor over other tyrosine kinase receptors, such as insulin receptor or Csk, KDR, lck, or zap. In a preferred embodiment, the inhibitor has a K_i 0.1 fold or less for a Tie-2 receptor than for an insulin receptor or Csk. In a more preferred embodiment, the inhibitor has K_i 0.01 fold or less for a Tie-2 receptor than for an insulin receptor or Csk. In a most preferred embodiment, the inhibitor has an K_i 0.001 fold less or less for a Tie-2 receptor than for an insulin receptor or Csk.

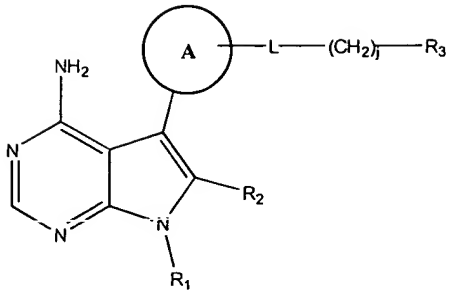
In a preferred embodiment, the Tie-2 inhibitor of the invention comprises two or more of the following when present at, or bound to, the Tie-2 catalytic domain: (a) a hydrogen bond donor positioned to interact with Glu 903 of human Tie-2; (b) a hydrogen bond acceptor positioned to interact with Ala 905 of human Tie-2; (c) a hydrogen bond donor positioned to interact with Ala 905 of human Tie-2; (d) a hydrophobic moiety positioned to interact with one or more of Ile 830, Val 838, Ala 853, Ile 886, Ile 902, Tyr 904, Ala 905 and Leu 971 of human Tie-2; (e) a hydrogen bond donor or positively charged functional group positioned to interact with Asp 912 of human Tie-2; (f) a hydrogen bond donor or hydrogen bond acceptor postioned to interact with Asn 909 of human Tie-2; (g) a hydrophobic moiety positoned to interact with one or more of Val 838, Lys 855, Ile 886, Ile 902, Leu 971 and Ala 981 of human Tie-2; (h) a hydrogen bond acceptor or negatively charged functional group positioned to interact with Lys 855 of human Tie-2; (i) a hydrogen bond acceptor positioned to interact with Asp 982 of human Tie-2; (j) a hydrogen bond acceptor positioned to interact with Phe 983 of human Tie-2; (k) a hydrophobic moiety positioned to interact with one or more of Leu 873, Leu 876, Ile 885, Ile 886, Leu 888, Leu 900, Ile 902, Ala 981 and Phe 983 of human Tie-2; (l) a hydrogen bond donor or positively charged functional group positioned to interact with Asp 982 of human Tie-2; (m) a hydrogen bond donor positioned to interact with Ile 886 of human Tie-2; (n) a hydrogen bond donor positioned to interact with Leu 768 of human Tie-2; (o) a hydrogen bond acceptor positioned to interact with Gly 831 of human Tie-2;

(p) a hydrogen bond donor or positively charged functional group positioned to interact with Glu 832 of human Tie-2; (q) a hydrogen bond acceptor or negatively charged functional group positioned to interact with Lys 840 of human Tie-2; (r) a hydrogen bond acceptor or negatively charged functional group positioned to interact with Lys 916 of human Tie-2; (s) a hydrogen bond acceptor or negatively charged functional group positioned to interact with Arg 968 of human Tie-2; (t) a hydrogen bond donor positioned to interact with Arg 968 of human Tie-2.

In preferred embodiments, the Tie-2 inhibitors of the invention comprise (b) and (d); (d) and at least one of (a), (b) and (c); (d) and at least two of (a), (b) and (c); (d) and at least two of (a), (b) and (c), and at least one of (e) and (f); (d) and (g), and at least two of (a), (b) and (c); (d), (g), at least two of (a), (b) and (c) and at least one of (e) and (f); (d), (g), (k), and at least two of (a), (b) and (c); (d), (g), (k), at least one of (e) and (f), at least two of (a), (b), and (c); (d), at least one of (i) and (j), and at least two of (a), (b) and (c); (d) and at least two of (a), (b) and (c), at least one of (e) and (f), and at least one of (i) and (j); (d), (g), (k), at least one of (i) and (j), and at least two of (a), (b) and (c); and (d), (g), (k), at least one of (e) and (f), and at least two of (a), (b) and (c).

Preferred Tie-2 inhibitors of the invention comprise a molecular scaffold or framework, to which the moieties and/or functional groups which interact with the Tie-2 subsites are attached, either directly or via an intervening moiety. The scaffold can be, for example, a peptide or peptide mimetic backbone, a cyclic or polycyclic moiety, such as a monocyclic, bicyclic or tricyclic moiety, and can include one or more hydrocarbonyl or heterocyclic rings. The molecular scaffold presents the attached interacting moieties in the proper configuration or orientation for interaction with the appropriate residues of Tie-2.

Pyrrolopyrimidines, such as inhibitor, I, II, III or IV, are preferred Tie-2 inhibitors. Methods for synthesizing pyrrolopyrimidines are described in PCT application number WO99/21560, the teachings of which are incorporated herein by reference in their entirety. In one embodiment, the inhibitors of the invention do not include the pyrrolopyrimidines represented by structural formula V:



V.

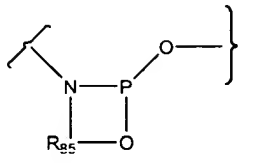
and pharmaceutically acceptable salts thereof, wherein:

Ring A is a six membered aromatic ring or a five or six membered heteroaromatic ring which is optionally substituted with one or more substituents selected from the group consisting of a substituted or unsubstituted aliphatic group, a halogen, a substituted or unsubstituted aromatic group, substituted or unsubstituted heteroaromatic group, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aralkyl, substituted or unsubstituted heteroaralkyl, cyano, nitro, $-NR_4R_5$, $-C(O)_2H$, -OH, a substituted or unsubstituted alkoxycarbonyl, $-C(O)_2$-haloalkyl, a substituted or unsubstituted alkylthio ether, a substituted or unsubstituted alkylsulfoxide, a substituted or unsubstituted alkylsulfone, a substituted or unsubstituted arylthio ether, a substituted or unsubstituted arylsulfoxide, a substituted or unsubstituted arylsulfone, a substituted or unsubstituted alkyl carbonyl, -C(O)-haloalkyl, a substituted or unsubstituted aliphatic ether, a substituted or unsubstituted aromatic ether, carboxamido, tetrazolyl, trifluoromethylsulphonamido, trifluoromethylcarbonylamino, a substituted or unsubstituted alkynyl, a substituted or unsubstituted alkyl amido, a substituted or unsubstituted aryl amido, $-NR_{95}C(O)R_{95}$, a substituted or unsubstituted styryl and a substituted or unsubstituted aralkyl amido, wherein R_{95} is an aliphatic group or an aromatic group;

L is -O-; -S-; -S(O)-; -$S(O)_2$-; -N(R)-; -N(C(O)OR)-; -N(C(O)R)-; -$N(SO_2R)$; -CH_2O-; -CH_2S-; -$CH_2N(R)$-; -CH(NR)-;-$CH_2N(C(O)R)$)-; -$CH_2N(C(O)OR)$-; -$CH_2N(SO_2R)$-; -CH(NHR)-; -CH(NHC(O)R)-; -$CH(NHSO_2R)$-; -CH(NHC(O)OR)-;-CH(OC(O)R)-;-CH(OC(O)NHR)-; -CH=CH-; -C(=NOR)-; -C(O)-; -CH(OR)-;-C(O)N(R)-; -N(R)C(O)-; -N(R)S(O)-;-$N(R)S(O)_2$-; -$OC(O)N(R)$-;-$N(R)C(O)N(R)$-; -NRC(O)O-;-$S(O)N(R)$-;-$S(O)_2N(R)$-; $N(C(O)R)S(O)$-; $N(C(O)R)S(O)_2$-; -$N(R)S(O)N(R)$-; -$N(R)S(O)_2N(R)$-; -C(O)N(R)C(O)-; -$S(O)N(R)C(O)$-; -$S(O)_2N(R)C(O)$-; -$OS(O)N(R)$-; -$OS(O)_2N(R)$-; -N(R)S(O)O-; -$N(R)S(O)_2O$-; -N(R)S(O)C(O)-; -$N(R)S(O)_2C(O)$-; -SON(C(O)R)-; -$SO_2N(C(O)R)$-; -N(R)SON(R)-; -$N(R)SO_2N(R)$-; -C(O)O-; -N(R)P(OR')O-; -N(R)P(OR')-; -N(R)P(O)(OR')O-; -N(R)P(O)(OR')-; -N(C(O)R)P(OR')O-; -N(C(O)R)P(OR')-; -N(C(O)R)P(O)(OR')O- or -N(C(O)R)P(OR')-, wherein R and R' are each, independently, -H, an acyl group, a substituted or unsubstituted aliphatic group, a substituted or unsubstituted aromatic group, a substituted or unsubstituted heteroaromatic group, or a substituted or unsubstituted cycloalkyl group; or

L is –$R_bN(R)S(O)_2$-, -$R_bN(R)P(O)$-, or -$R_bN(R)P(O)O$-, wherein R_b is an alkylene group which when taken together with the sulphonamide, phosphinamide, or phosphonamide group to which it is bound forms a five or six membered ring fused to ring A; or

L is represented by one of the following structural formulas:



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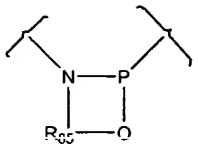

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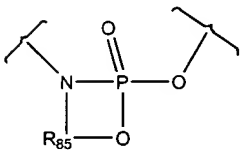

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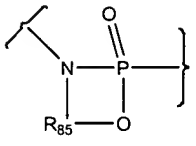

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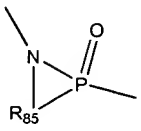
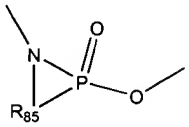


or


wherein R_{85} taken together with the phosphinamide, or phophonamide is a 5-, 6-, or 7-membered, aromatic, heteroaromatic or heterocycloalkyl ring system;

R_1 is a substituted aliphatic group, a substituted cycloalkyl, a substituted bicycloalkyl, a substituted cycloalkenyl, an optionally substituted aromatic group, an optionally substituted heteroaromatic group, an optionally substituted heteroaralkyl, an optionally substituted heterocycloalkyl, an optionally substituted heterobicycloalkyl, an optionally substituted alkylamindo, and optionally substituted arylamido, an optionally substituted -$S(O)_2$-alkyl or optionally substituted -$S(O)_2$-cycloalkyl, a -C(O)-alkyl or an optionally substituted –C(O)-alkyl, provided that when R_1 is an aliphatic group or cycloalkyl group, R_1 is not exclusively substituted with one or more substitutent selected from the group

consisting of hydroxyl and lower alkyl ethers, provided that the heterocycloalkyl is not 2-phenyl-1,3-dioxan-5-yl and provided that an aliphatic group is not substituted exclusively with one or more aliphatic groups, wherein one or more substituent is selected from the group consisting of a substituted or unsubstituted aliphatic group, a substituted or unsubstituted aromatic group, a substituted or unsubstituted heteroaromatic, a substituted or unsubstituted aralkyl, a substituted or unsubstituted heteroaralkyl, a substituted or unsubstituted cycloalkyl, a substituted or unsubstituted heterocycloalkyl, a substituted or unsubstituted aromatic ether, a substituted or unsubstituted aliphatic ether, a substituted or unsubstituted alkoxycarbonyl, a substituted or unsubstituted alkylcarbonyl, a substituted or unsubstituted arylcarbonyl, a substituted or unsubstituted heteroarylcarbonyl, substituted or unsubstituted aryloxycarbonyl, -OH, a substituted or unsubstituted aminocarbonyl, an oxime, a substituted or unsubstituted azabicycloalkyl, heterocycloalkyl, oxo, aldehyde, a substituted or unsubstituted alkyl sulfonamido group, a substituted or unsubstituted aryl sulfonamido group, a substituted or unsubstituted bicycloalkyl, a substituted or unsubstituted heterobicycloalkyl, cyano, $-NH_2$, an alkylamino, ureido, thioureido and –B-E;

B is a substituted or unsubstituted cycloalkyl, a substituted or unsubstituted heterocycloalkyl, a substituted or unsubstituted aromatic, a substituted or unsubstituted heteroaromatic, an alkylene, an aminoalkyl, an alkylenecarbnonyl, or an aminoalkylcarbonyl;

E is a substituted or unsubstituted azacycloalkyl, a substituted or unsubstituted azacycloalkylcarbonyl, a substituted or unsubstituted azacycloalkylsulfonyl, a substituted or unsubstituted azacycloalkylalkyl, a substituted or unsubstituted heteroaryl, a substituted or unsubstituted heteroarylcarbonyl, a substituted or unsubstituted heteroarylsulfonyl, a substituted or unsubstituted heteroaralkyl, a substituted or unsubstituted alkyl sulfonamido, a substituted or unsubstituted aryl sulfonamido, a substituted or unsubstituted

bicycloalkyl, a substituted or unsubstituted ureido, a substituted or unsubstituted thioureido or a substituted or unsubstituted aryl;

R_2 is –H, a substituted or unsubstituted aliphatic group, a substituted or unsubstituted cycloalkyl, a halogen, -OH, cyano, a substituted or unsubstituted aromatic group, a substituted or unsubstituted heteroaromatic group, a substituted or unsubstituted heterocycloalkyl, a substituted or unsubstituted aralkyl, a substituted or unsubstituted heteroaralkyl, $-NR_4R_5$, or $-C(O)NR_4R_5$;

R_3 is a substituted or unsubstituted aliphatic group, a substituted or unsubstituted alkenyl group, a substituted or unsubstituted cycloalkyl, a substituted or unsubstituted aromatic group, a substituted or unsubstituted heteroaromatic group, or a substituted or unsubstituted heterocycloalkyl;

provided that L is $-SN(R)-$, $-S(O)N(R)-$, $-S(O)_2N(R)-$, $-N(R)S-$, $-N(R)S(O)-$, $-N(R)S(O)_2-$, $-N(R)SN(R')-$, $-N(R)S(O)N(R')-$, or $-N(R)S(O)_2N(R')-$ when R_3 is a substituted or unsubstituted aliphatic group, a substituted or unsubstituted alkenyl group;

provided that j is 0 when L is –O-, $–CH_2NR-$, -C(O)NR- or –NRC(O)- and R_3 is azacycloalkyl or azaheteroaryl; and

provided that j is 0 when L is –O- and R_3 is phenyl;

R_4, R_5 and the nitrogen atom together form a 3, 4, 5, 6 or 7-membered, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted heterobicycloalkyl or a substituted or unsubstituted heteroaromatic; or

R_4 and R_5 are each, independently, -H, azabicycloalkyl, heterocycloalkyl, a substituted or unsubstituted alkyl group or Y-Z;

Y is selected from the group consisting of $-C(O)-$, $-(CH_2)_p-$,$-S(O)_2-$, $-C(O)O-$, $-SO_2NH-$, -CONH-, $(CH_2)_pO-$, $-(CH_2)_pNH-$, $-(CH_2)_pS-$, $-(CH_2)_pS(O)-$, and $-(CH_2)_pS(O)_2-$;

p is an integer from 0 to 6;

Z is –H, a substituted or unsubstituted alkyl, substituted or unsubstituted amino, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl or

substituted or unsubstituted heterocycloalkyl group; and

j an integer from 0 to 6.

As used herein, aromatic groups include carbocyclic ring systems (e.g. phenyl and cinnamyl) and fused polycyclic aromatic ring systems (e.g. naphthyl and 1,2,3,4-tetrahydronaphthyl). Arromatic groups are also referred to as aryl groups herein.

Heteroaromatic groups, as used herein, include heteroaryl ring systems (e.g., thienyl, pyridyl, pyrazole, isoxazolyl, thiadiazolyl, oxadiazolyl, indazolyl, furans, pyrroles, imidazoles, pyrazoles, triazoles, pyrimidines, pyrazines, thiazoles, isoxazoles, isothiazoles, tetrazoles, or oxadiazoles) and heteroaryl ring systems in which a carbocyclic aromatic ring, carbocyclic non-aromatic ring or heteroaryl ring is fused to one or more other heteroaryl rings (e.g., benzo(b)thienyl, benzimidazole, indole, tetrahydroindole, azaindole, indazole, quinoline, imidazopyridine, purine, pyrrolo[2,3-d]pyrimidine, pyrazolo[3,4-d]pyrimidine) and their N-oxides.

An aralkyl group, as used herein, is an aromatic substituent that is linked to a compound by an aliphatic group having from one to about six carbon atoms.

An heteroaralkyl group, as used herein, is a heteroaromatic substituent that is linked to a compound by an aliphatic group having from one to about six carbon atoms.

A heterocycloalkyl group, as used herein, is a non-aromatic ring system that has 3 to 8 atoms and includes at least one heteroatom, such as nitrogen, oxygen, or sulfur.

An acyl group, as used herein, is an $-C(O)NR_xR_z$, $-C(O)OR_x$, $-C(O)R_x$, in which R_x and R_z are each, independently, -H, a substituted or unsubstituted aliphatic group or a substituted or unsubstituted aromatic group.

As used herein, aliphatic groups include straight chained, branched or cyclic C_1-C_8 hydrocarbons which are completely saturated or which contain one or more units of unsaturation. A "lower alkyl group" is a saturated aliphatic group having form 1-6 carbon atoms.

Inhibitor I bound to the catalytically inactive mutant of Tie-2 (see Fig. 2 for sequence and Fig. 3 for atomic coordinates) crystallized in the space group C2221. The x-ray crystallographic structure reveiled the following interactions:

The pyrrolopyrimidine ring of the inhibitor I forms hydrogen bonds to residues in the hinge region and interacts with purine core region. The core of the inhibitor presents a hydrogen bond donor in the form of the amino proton of the 4-NH_2 substituent to the carbonyl oxygen of E903. Atom N3 of the pyrimidine ring accepts a hydrogen bond from the backbone N-H of A905. The ring system of the core presents a planar face to residues of both the C-terminal and N-terminal lobes. The residues in these areas present a hydrophobic surface which "sandwiches" the planar core of the inhibitor. Residues involved in this hydrophobic sandwich region include I830, V838, I86, I902 and L971. Atoms N1 and N7 of the core face the solvent exposed mouth of the binding pocket. Atom C6 faces the long axis of the nucleotide binding loop of the N-terminal lobe of the protein.

The N7 cyclopentane ring is directed towards solvent but is still within the protein cavity. This region was described above as the extended sugar pocket after the binding mode of the ribose ring of ATP observed in other kinase structures. This region is characterized by hydrophobic interactions with primarily I830 and L971. Methylene groups of E832 may also contribute in this fashion.

The phenyl ring attached to C5 of the pyrrolopyrimidine ring is in a predominantly hydrophobic area, generated by residues from the purine core region, the distal hydrophobic pocket and methylene groups from the catalytic lysine, K855. The hydrophobic contacts are with residues V838, I886, I902, L971 and A981. Lysine 855 is highly mobile, so it is also possible that the Cl atom meta to the pyrrolopyrimidine ring is receiving a hydrogen bond.

The sulfonamide linker makes a clear hydrogen bond with an amide proton of D982 and may also make a hydrogen bond to the amide proton of F983.

The terminal phenyl ring (labelled ring C) is located in the distal hydrophobic pocket. Primary contacts are with L876, I886, L888 and F983.

Inhibitor II bound to the catalytically inactive mutant of Tie-2 (see Fig. 2 for sequence and Fig. 4 for atomic coordinates) crystallized in the space group P42212. The x-ray crystallographic structure reveiled the following additional interactions:

The pyrrolopyrimidine core, B-ring, linker and C-ring bind the same way as inhibitor I. The N-7 cyclohexyl N-methy piperazinyl group occupies the extended sugar pocket and makes a strong ionic interaction with D912.

Inhibitor III bound to the catalytically inactive mutant of Tie-2 (see Fig. 2 for sequence and Fig. 4 for atomic coordinates) crystallized in the space group P42212. The x-ray crystallographic structure reveiled the following additional interactions:

The pyrrolopyrimidine core binds the same way as inhibitor I. The N-7 cyclohexyl N-methy piperazinyl group occupies the extended sugar pocket and makes a strong ionic interaction with D912 as in Tie-2/inhibitor II. The B-ring binds in a similar fashion to inhibitor I, however, the hydrogen bond between a halogen, fluorine in this case, and K855 is more clear. The linker makes two clear hydrogen bonds to backbone amide protons of D983 and F983. The C-ring occupies the distal hydrophobic pocket with main interactions coming from L876, I886, L888, L900, I902 and F983.

Inhibitor IV bound to the catalytically inactive mutant of Tie-2 (see Fig. 2 for sequence and Fig. 4 for atomic coordinates) crystallized in the space group P42212. The x-ray crystallographic structure reveiled the following additional interactions:

The pyrrolopyrimidine core binds the same way as inhibitor I. The N-7 cyclohexyl N-methy piperazinyl group occupies the extended sugar pocket and makes a strong ionic interaction with D912 as in Tie-2/inhibitor II. The B-ring binds in a similar fashion to inhibitor I, however there is no chlorine atom to act as a potential hydrogen bond partner. The linker in this case is an oxygen atom which accepts a hydrogen bond from the catalytic lysine, K855. The C-ring occupies the distal hydrophobic pocket with main interactions coming from L876, I886, I902 and F983.

In one embodiment, the present invention relates to a method of treating a Tie-2-dependent condition in a patient. The method comprises the step of administering to the patient a therapeutically effective amount of a Tie-2 inhibitor as described above. The patient can be any animal, and is, preferably, a mammal and, more preferably, a human.

A "Tie-2-dependent condition" is a disease or medical condition in which the catalytic activity of Tie-2 plays a role, for example, in the development of the disease or

condition. For example, in one embodiment, the condition is characterized by excessive vascular proliferation. Tie-2 inhibitors are useful in treating angiogenesis dependent disorders, and disorders involving aberrant endothilial-pereindothelial interactions (e.g., restenosis).

Tie-2 dependent conditions include hyperproliferative disorders, cancer, a cardiovascular condition, an ocular condition, von Hippel Lindau disease, pemphigoid, psoriasis, Paget's disease, polycystic kidney disease, fibrosis, sarcoidosis, cirrhosis, thyroiditis, Osler-Weber-Rendu disease, chronic inflammation, synovitis, inflammatory bowel disease, Crohn's disease, rheumatoid arthritis, osteoarthritis, psoriatic arthritis, an ulcer and sepsis. In addition a Tie-2 inhibitor can be used to decrease fertility in a patient.

Preferred methods of treatment are where the cancer is a solid tumor, a sarcoma, fibrosarcoma, osteoma, melanoma, retinoblastoma, a rhabdomyosarcoma, glioblastoma, neuroblastoma, teratocarcinoma, an hematopoietic malignancy, malignant ascites, Kaposi's sarcoma, Hodgkin's disease, lymphoma, myeloma or leukemia.

Another preferred method of treatment is where the cardiovascular condition, atherosclerosis, restenosis, ischemia/reperfusion injury, chronic occlusive pulmonary disease, vascular occlusion, carotid obstructive disease, Crow-Fukase (POEMS) syndrome, anemia, ischemia, infarct, vascular leakage disorders.

Yet another preferred method of treatment is where the ocular condition is ocular or macular edema, ocular neovascular disease, scleritis, radial keratotomy, uveitis, vitritis, myopia, optic pits, chronic retinal detachment, post-laser treatment complications, conjunctivitis, Stargardt's disease, Eales disease, retinopathy, macular degeneration or microangiopathy.

A Tie-2 inhibitor can also be used in a method of promoting angiogenesis or vasculogenesis. In addition, a Tie-2 inhibitor can be administered with a pro-angiogenic growth factor.

A therapeutically effective amount, as this term is used herein, is an amount which results in partial or complete inhibition of disease progression or symptoms. Such an amount will depend, for example, on the size and gender of the patient, the condition to

be treated, the severity of the symptoms and the result sought, and can be determined by one skilled in the art.

The compound of the invention can, optionally, be administered in combination with one or more additional drugs or therapies which, for example, are known for treating and/or alleviating symptoms of the condition mediated by Tie-2. The additional drug can be administered simultaneously with the compound of the invention, or sequentially. For example, the Tie-2 inhibitor can be administered in combination with another anticancer agent, as is known in the art. Additional therapies which may be coadministered would include, for example, radiation therapy, ultraviolet irradiation, hyperthermia, laser irradiation, targeted radionuclides and neutron bombardment.

The invention further provides pharmaceutical compositions comprising one or more of the Tie-2 inhibitors described above. Such compositions comprise a therapeutically (or prophylactically) effective amount of one or more Tie-2 binding inhibitors, as described above, and a pharmaceutically acceptable carrier or excipient. Suitable pharmaceutically acceptable carriers include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The carrier and composition can be sterile. The formulation should suit the mode of administration.

Suitable pharmaceutically acceptable carriers include but are not limited to water, salt solutions (e.g., NaCl), alcohols, gum arabic, vegetable oils, benzyl alcohols, polyethylene glycols, gelatin, carbohydrates such as lactose, amylose or starch, cyclodextrin, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid esters, hydroxymethylcellulose, polyvinyl pyrolidone, etc. The pharmaceutical preparations can be sterilized and if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, flavoring and/or aromatic substances and the like which do not deleteriously react with the active compounds.

The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The

composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, polyvinyl pyrrolidinone, sodium saccharine, cellulose, magnesium carbonate, etc.

The composition can be formulated in accordance with the routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachet indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water, saline or dextrose/water. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The pharmaceutical compositions of the invention can also include an agent which controls release of the Tie-2 inhibitor compound, thereby providing a timed or sustained release composition.

The Tie-2 inhibitor can be administered subcutaneously, intravenously, parenterally, intraperitoneally, intradermally, intramuscularly, intraocularly, topically, enteral (e.g., orally), rectally, nasally, buccally, sublingually, vaginally, by inhalation spray, by drug pump or via an implanted reservoir in dosage formulations containing conventional non-toxic, physiologically acceptable carriers or vehicles. The preferred method of administration is by oral delivery. The form in which it is administered (e.g., syrup, elixir, capsule, tablet, solution, foams, emulsion, gel, sol) will depend in part on the route by which it is administered. For example, for mucosal (e.g., oral mucosa, rectal, ocular mucosa, intestinal mucosa, bronchial mucosa) administration, nose drops, aerosols, inhalants, nebulizers, eye drops or suppositories can be used. The compounds

and agents of this invention can be administered together with other biologically active agents, such as analgesics, anti-inflammatory agents, anesthetics and other agents which can control one or more symptoms or causes of a Tie-2 dependent condition.

In a specific embodiment, it may be desirable to administer the agents of the invention locally to a localized area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, transdermal patches, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes or fibers. For example, the agent can be injected into the joints.

EXAMPLES

Example 1 Protein Purification

$(His)_6$Tie-2 802-1124, D964N, which contains a TEV protease cleavage peptide, was expressed recombinantly by baculovirus infection of SF-9 cells. Cells were lysed in a buffer containing 20 mM Tris pH 8.0, 137 mM NaCl , 10 % glycerol, 1 % Triton X-100, 1 mM ADP, 5 mM $MgCl_2$ and complete protease inhibitor, EDTA-free cocktail from Boehringer Mannhein.. The ligand ADP/Mg^{++} was maintained at this concentration in buffers of all subsequent purification steps. The cell lysate was centrifuged and the supernatant was applied to a Ni^{++} chelating sepharose column which had been equilibrated in 50 mM HEPES, pH 7.5, 0.3 M NaCl. Tie-2 was eluted by competition with 100 mM imidazole. The eluted $(His)_6$ Tie-2 was digested with Tev protease and dialyzed against 50 mM HEPES, pH 7.5, 0.25 M NaCl, 5 mM DTT. The dialyzed sample was centrifuged to remove any precipitated protein, and Tie-2 was bound to a MonoQ anion exchange column and eluted with a linear 20 column volume gradient of 0.025-0.2 M NaCl. Typically, differences in the monodispersity of early eluting verses late eluting fractions could be detected using Dynamic Light Scattering (DLS). Sample purity was

assessed with SDS-PAGE, native PAGE, and LC/MS total mass analysis. Fractions with similar DLS characteristics were pooled and concentrated to greater than 2 mg/ml using ultrafiltration at –80 ^{0}C. The ultracentrifuged samples were used in crystallographic experiments described below. Table I lists a range of conditions suitable for crystallization.

Example 2:

I. Diphosphorylated Tie-2 802-1124

A. Crystallization Conditions:

Tie-2 802-1124 ($2PO_4$) protein was crystallized in a sitting or hanging drop geometry using a vapor diffusion method. The protein concentration was 5 mg/ml, and the well solution was 10% PEG 6,000; 0.1 M HEPES, pH 7.5; 5% MPD (2-methyl-2,4-pentanediol). Drops were set up using equal volumes of protein and well solution containing 500 μM inhibitor. Crystals routinely grew to 0.4 mm x 0.1 mm x 0.01 mm in about a week. Crystals were of the space group P2(1)2(1)2(1) with unit cell dimensions a = 54.320 Å, b = 75.872 Å, c = 78.143 Å , and $\alpha = \beta = \gamma = 90.0^{\circ}$. Table I list a range of conditions which are suitable for crystallization.

B. Data Collection

Data on ligand bound crystals were collected on a Rigaku RU300 rotating anode generator running at 50kV 150 mA equipped with an R-Axis II phosphoimage plate detector. X-rays were monochromatized by long mirrors and filtered with a 0.0067μm Nickel filter. Data were processed and reduced with DENZO and SCALEPACK (Minor, W. 1993). Data were collected to 3.5 Å resolution.

C. Data Processing

Programs in the CCP4 suite (Collaborative Computational Project, Number 4

1994) (tomtz, trunc, cad and ecalc) were used to format and process the data for molecular replacement. The molecular replacement program AMORE (Navaza, J. 1994) was used successfully to find phases for the data set using an initial model. The initial model was composed of the carboxy-terminal portion (residues 566-575 and 592-672) of the FGFR kinase domain trimmed back to poly-Alanine (PDB accession number 1FGK). A second round of AMORE with a more complete model (residues 464-485, 491-500, 505-575 and 592-762) was also performed to confirm the phasing solution.

D. Optimization of Model

Several round of least-squared minimization using CNS (Brunger, A.T. et al., 1998) alternating with manual rebuilding, using the graphics program O, version 6.2.1 (Jones, A., 1997; Kleywegt G. J., 1995) were performed iteratively to improve the model while comparing it to electron density maps generated after each round with coefficients 2fo-fc contoured at a level of 1.0 sigma.

II. Tie-2 (D964N) 802-1124 (SEQ ID NO 2)

A. Crystallization Conditions

Purified Tie-2 (D964N) 802-1124 protein was crystallized in a sitting drop geometry using the vapor diffusion method. The protein concentration was 2.5 mg/ml, and the well solution was 1.0 to 1.5 M ammonium sulfate, 0.1M MES, pH 6.5, 5% dioxane (1,4-dioxane). Drops were set up using equal volumes of protein and well solution containing 100-300 μM inhibitor. Crystals routinely grew to 0.3 mm x 0.05 mm x 0.01 mm in about 2-3 days. Crystals of Tie-2/inhibitor I were of the space group C222(1) with unit cell dimensions a = 75.195 Å, b = 116.287 Å, c = 95.060 Å and $\alpha = \beta = \gamma$ - 90.0°. Crystals of the Tie-2/inhibitor II, III or IV complex were of the space group P42212 with unit cell dimensions a = b = 86.0 Å, c = 112.0 Å and $\alpha = \beta = \gamma$ - 90.0°.

B. Data Collection

Data on a ligand-bound crystal (Tie-2 (D964N) 802-1124) complexed with inhibitors I, II, III, or IV were collected at the beamline X25 at Brookhaven National Laboratory (Upton, NY) equipped with the Brandeis B4, CCD detector. Data were processed and reduced with DENZO and SCALEPACK (Minor, W. 1993). Data for the Tie-2/inhibitor I complex were collected complete to 2.75 Å resolution, with higher resolution reflections visible to 2.0 Å resolution.

C. Data Processing

Programs in the CCP4 suite (Collaborative Computational Project, Number 4 1994) (tomtz, trunc, cad and ecalc) were used to format and process the data for molecular replacement. The molecular replacement program AMORE (Navaza, J. 1994) was used successfully to find phases for the data set using an initial model. The initial model was composed of the a conservative portion of the FGFR kinase domain (Tie2 residue numbering 818-830, 841-842, 850-857, 866-890, 900-916, 935-981, 1001-1093). The model, mostly poly-Alanine, was trimmed of loop regions which diverged upon superposition of five tyrosine kinase structures (IRK, HCK, SRC, FGFR, and LCK). In addition this model included only those side-chain residues in positions where an identical side-chain was found in the FGFR model.

D. Optimization of Model

Several round of least-squared minimization using CNS (Brunger, A. T. et al., 1998) alternating with manual rebuilding, using the graphics program O, version 6.2 (Jones, A., 1997; Kleywegt G. J., 1995) were performed to iteratively improve the model while comparing it to two electron density maps: one generated with coefficients 2fo-fc contoured at a level of 1.0 sigma and the other generated with coefficients fo-fc contoured at a level of 1.5 sigma.

E. Inhibitor Docking

Inhibitor I was found to be bound to the active site. It was initially docked by hand in O by visually inspecting the electron density maps and adjusting the torsion angles of the inhibitor. Parameter and topology files were generated for CNS using the X-util program xplo2d (Kleywegt G. J. and Jones, T.A. 1997) and modified slightly to properly model chlorine in the inhibitor.

III. Tie-2 (D964N) 802-1124 (SEQ ID NO 2)

A. Crystallization Conditions

The protein (construct Tie-2D964N) was provided in a buffer containing 25mM HEPES, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 1 mM ADP and 5 mM DTT. The protein concentration was about 2.3 mg/ml as determined with a Coomassie Plus assay, BSA as standard.
The inhibitor III was dissolved in DMSO to give a 50 mM stock solution. Stock solution was added to the protein solution for a final inhibitor concentration of 2mM.
Crystallization conditions were screened with Hampton Screen *Crystal screen, Crystal screen2, Membfac, Natrix* and *PEG/ion screen* at room temperature and 4°C.
Crystals grew with precipitation buffer: 20% PEG 3350, 0,2M tri-Lithium Citrat pH 8,1 (Hampton Screen *PEG/ion screen*, Nr. 45) sitting or hanging drop: 750µl buffer in reservoir in the drop typically 1µl - 2µl protein and 1µl - 2µl reservoir solution were mixed.
Addition of the following additives (10% by volume to the drop) also yielded crystals:

Add. Screen I Nr.:01	0.1M Ba-Chloride
Add. Screen I Nr.:03	0.1M Ca-Chloride
Add. Screen I Nr.:06	0.1M Mg-Chloride
Add. Screen I Nr.:16	0.1M Trimehylamine
Add. Screen I Nr.:22	30% Ethanol

Add. Screen II Nr.:08	30% Xylitol
Add. Screen II Nr.:13	30% 1,5Diaminopentan-dihydrochloride
Add. Screen II Nr.:14	30% 1,8 Diaminooctane
Add. Screen II Nr.:17	0.1M Hexaaminocobalt-trichloride
Add. Screen III Nr.:02	1.0M Cesium-chloride
Add. Screen III Nr.:04	1.0M Lihium-chloride
Add. Screen III Nr.:06	0.5M Sodium-flouride
Add. Screen III Nr.:16	40% Acetonitrile
Add. Screen III Nr.:18	40% n-Propanol
Add. Screen III Nr.:19	5% Ethyl-acetate
Add. Screen III Nr.:20	40% Acetone
Add. Screen III Nr.:21	2,5% Dichlormethane
Add. Screen III Nr.:22	7% n-Butanol
Add. Screen III Nr.:24	0.1M 1,4 Dithio-DL-threitol

B. Data collection:

Data were measured at the beam line BW 6 of the Max-Planck-Society at DESY, Hamburg.

The crystals were shock cooled to 100 K; cryobuffer was crystallization buffer plus 20 – 30 % glycerol. 213 frames with delta phi=0.25 degrees were collected with a MAR CCD detector, at a crystal detector distance of 120mm and a wavelength of 1.072Å.

Crystals are of a tetragonal space group with unit cell dimensions a=b=86.0 Å and c=112.0 Å. The cell dimensions of different crystals vary (for a and b between 85 and 87Å, for c between 97 and 113). Extinctions indicate the space group P42212 which was confirmed by molecular replacement.

Table II: Crystallization conditions for Tie-2/inhibitor complexes.

Condition	Tie-2 802-1124 D964N	Tie-2 802-1124 (diphosphorylated)
Protein concentration	2.5 mg/mL optimal range 1.5 – 4 mg/mL limits 1.0 – 5.0 mg/mL	5 mg/mL optimal range 2.5 – 10 mg/mL
Buffer concentration	100 mM MES optimal range 50–250 mM Limits 20–300 mM	100 mM HEPES optimal Range 50-150 mM Limits 20-300 mM
pH	6.5 optimal range 5.5 – 7.5	7.5 optimal range 7.0 – 7.7 limits 6.5 – 8.0
Buffer Identity	Buffers capable of buffering in a similar pH range expected to give similar results	(same)
Precipitant	$(NH_4)_2SO_4$ Range 1.0 – 1.5 M Limits 0.7 – 1.8 M	10% PEG 6000 optimal conc. range 5-15% conc. limits 1 – 20% MW range 4000 – 8000 MW limits may be much wider
Additive parameters	5% 1,4-dioxane optimal range 0 – 10% (higher concentrations etch the plastic vessel in which the experiment is done; higher concentrations may be possible in a resistant vessel) 1,3-dioxane, similar molecules, or mixtures in various ratios should also give similar results	5% MPD (2-Me-2,4-pentanediol) optimal range 0 – 10%

Additive identities	Examples which have been successfully added: 1,5-diaminopentane Glycerol (1-10%) Ethylene glycol (1-10%) Spermidine (10 – 300 mM) Combinations, in varying ratios, may give similar results	(same)
Drop volumes and ratios	2 μL protein + 2 μL well solution optimal Total volume range: up to 200 μL, assuming a sitting geometry for larger volumes Volume Ratio range: 1 part protein to 0.5 – 2.0 parts well solution	(same)
Well volume (for 4 μL crystallization drop)	Range 500 – 1000 μL Limits 250 – large volume (limited by the distance between the drop and the surface of the well solution allowed by the vessel geometry, see below)	(same)
Drop – well solution distance	2 cm optimal Range 1-4 cm Limits: 0.1 cm - 5 cm	(same)
Temperature	room temp optimal (22 – 25 °C) limits 17 – 30 °C	(same)
Ligands	ADP/Mg^{2+} and analogs Inhibitors: inhibitors I-IV, analogs Expect similar results from ligands that bind reversibly under crystallization conditions with K_d values < 1 mM	(same)

Constructs	Variants in amino acid sequence that crystallize in the same space group and unit cell should be considered equivalent Additional constructs would include deletion of unstructured termini as determined by crystal structure of this construct. For example, deletion of the C-terminal 24 residues (leaving 802-1100 has been prepared, which is likely to yield similar results	(same)
Posttranslational modification	Variants in posttranslational modification that crystallize in the same space group and unit cell should be considered equivalent	2 phosphate forms have been crystallized. This protein contains one phosphate on either Y897 or Y899 and one on one of five Tyr residues, at amino acids 1012, 1015, 1024, 1040, and 1048 Other phosphorylated forms may give similar results. A single phosphate species has been observed in which the phosphate is on either Y897 or Y899 has also been isolated. In addition, 3 and 4 phosphate species have been isolated which may crystallize.
Space group	C222(1)	P2(1)2(1)2(1)

Unit cell	a = 75.195 Å, b = 116.287 Å, c = 95.060 Å Variations of ± 2% should be considered equivalent Angles: a = b = c = 90° Observed variations of ± 1% should be considered equivalent	a = 54.320 Å, b = 75.872 Å, c = 78.143 Å Variations of ± 2% should be considered equivalent Angles: a = b = c = 90° Observed variations of ± 1% should be considered equivalent
Other crystallization tricks that should give at least equivalent results	Low gravity Temperature oscillations Presence of cryoprotectant (15-25% glycerol added before data collection) Variations in crystallization tray geometry Data collection temperature (range: minus 180 to plus 25 °C)	(same)

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Example 3: *In Vitro* Potency Test of Tie-2 Inhibitors

The *in vitro* potency of compounds in inhibiting these protein kinases may be determined by the procedures detailed below.

The potency of compounds can be determined by the amount of inhibition of the phosphorylation of an exogenous substrate (e.g., synthetic peptide (Z. Songyang *et al.*, *Nature*. 373:536-539) by a test compound relative to control.

Human Tie-2 Kinase Production and Purification

The coding sequence for the human Tie-2 intra-cellular domain (aa775-1124) was generated through PCR using cDNAs isolated from human placenta as a template. A poly-His_6 sequence was introduced at the N-terminus and this construct was cloned into transfection vector pVL 1939 at the Xba 1 and Not 1 site. Recombinant BV was generated through co-transfection using the BaculoGold Transfection reagent (PharMingen). Recombinant BV was plaque purified and verified through Western analysis. For protein production, SF-9 insect cells were grown in SF-900-II medium at 2 x 106/ml, and were infected at MOI of 0.5. Purification of the His-tagged kinase used in screening was analogous to that described for KDR.

EGFR Tyrosine Kinase Source

EGFR was purchased from Sigma (Cat # E-3641; 500 units/50 l) and the EGF ligand was acquired from Oncogene Research Products/Calbiochem (Cat # PF011-100).

Enzyme Linked Immunosorbent Assay (ELISA) For PTKs

Enzyme linked immunosorbent assays (ELISA) were used to detect and measure the presence of tyrosine kinase activity. The ELISA were conducted according to known protocols which are described in, for example, Voller, *et al.,* 1980, "Enzyme-Linked Immunosorbent Assay," In: *Manual of Clinical Immunology, 2d ed.,* edited by Rose and Friedman, pp 359-371 Am. Soc. of Microbiology, Washington, D.C.

The disclosed protocol was adapted for determining activity with respect to a specific PTK. For example, preferred protocols for conducting the ELISA experiments is provided below. Adaptation of these protocols for determining a compound's activity for other members of the receptor PTK family, as well as non-receptor tyrosine kinases, are well within the abilities of those skilled in the art. For purposes of determining inhibitor selectivity, a universal PTK substrate (e.g., random copolymer of poly(Glu_4 Tyr), 20,000-50,000 MW) was employed together with ATP (typically 5 μM) at concentrations approximately twice the apparent Km in the assay.

The following procedure was used to assay the inhibitory effect of compounds of this invention on Tie-2 tyrosine kinase activity:

Buffers and Solutions:

PGTPoly (Glu,Tyr) 4:1

Store powder at -20°C. Dissolve powder in phosphate buffered saline (PBS) for 50mg/ml solution. Store 1ml aliquots at -20°C. When making plates dilute to 250 g/ml in Gibco PBS.

Reaction Buffer: 100mM Hepes, 20mM $MgCl_2$, 4mM $MnCl_2$, 5mM DTT, 0.02%BSA, 200μM $NaVO_4$, pH 7.10

ATP: Store aliquots of 100mM at -20°C. Dilute to 20μM in water

Washing Buffer: PBS with 0.1% Tween 20

Antibody Diluting Buffer: 0.1% bovine serum albumin (BSA) in PBS

TMB Substrate: mix TMB substrate and Peroxide solutions 9:1 just before use or use K-Blue Substrate from Neogen

Stop Solution: 1M Phosphoric Acid

Procedure

1. Plate Preparation:

Dilute PGT stock (50mg/ml, frozen) in PBS to a 250µg/ml. Add 125µl per well of Corning modified flat bottom high affinity ELISA plates (Corning #25805-96). Add 125µl PBS to blank wells. Cover with sealing tape and incubate overnight 37°C. Wash 1x with 250µl washing buffer and dry for about 2hrs in 37°C dry incubator.

Store coated plates in sealed bag at 4°C until used.

2. Tyrosine Kinase Reaction:

-Prepare inhibitor solutions at a 4x concentration in 20% DMSO in water.

-Prepare reaction buffer

-Prepare enzyme solution so that desired units are in 50µl, e.g. for KDR make to 1 ng/ l for a total of 50ng per well in the reactions. Store on ice.

-Make 4x ATP solution to 20µM from 100mM stock in water. Store on ice

-Add 50µl of the enzyme solution per well (typically 5-50 ng enzyme/well depending on the specific activity of the kinase)

-Add 25µl 4x inhibitor

-Add 25µl 4x ATP for inhibitor assay

-Incubate for 10 minutes at room temperature

-Stop reaction by adding 50µl 0.05N HCl per well

-Wash plate

**Final Concentrations for Reaction: 5µM ATP, 5% DMSO

3. Antibody Binding

-Dilute 1mg/ml aliquot of PY20-HRP (Pierce) antibody (a phosphotyrosine antibody) to 50ng/ml in 0.1% BSA in PBS by a 2 step dilution (100x, then 200x)

-Add 100µl Ab per well. Incubate 1 hr at room temp. Incubate 1 hr at 4C.

-Wash 4x plate

4. Color reaction

-Prepare TMB substrate and add 100µl per well

-Monitor OD at 650nm until 0.6 is reached

-Stop with 1M Phosphoric acid. Shake on plate reader.

-Read OD immediately at 450nm

Optimal incubation times and enzyme reaction conditions vary slightly with enzyme preparations and are determined empirically for each lot.

Example 4: Cellular Assay for Determining the Potency of Tie-2 Inhibitors

The following cellular assay can be used to determine the potency of a Tie-2 inhibitor.

"NIH-3T3/hTEK Cell line:

A retroviral expression vector containing the full length Tie-2 cDNA, LNCX6 h-TEK, was kindly provided to us by Dr. Kevin Peters. A tumorigenic subline of NIH 3T3 cells was transfected with 10 ìg of LNCX6 h-TEK by calcium phosphate precipitation method and selected with 400 ìg/ml neomycin. Individual clones were isolated and analyzed for the presence of Tie-2 protein by Western blotting. Maximum expression of Tie-2 was observed in clone #67. Expression of Angiopoietin 1 message has been shown using PCR and an autocrine loop is revealed in the presence of vanadate

Cellular Tie-2 assay:

Tie-2 cellular autophosphorylation was measured using the NIH-3T3/hTEK (hTEK) cell line. Cells were seeded in 96 well plates overnight. The media was removed and cells treated with inhibitor for 20 minutes and phosphotase inhibitor $NaVO_3$ (2mM) for 15 more minutes. Cells were lysed with RIPA buffer and lysates were immunoprecipitated using a specific a-Tie-2 monoclonal antibody (KP33, provided by Dr. Kevin Peters) and the IP'd protein run on SDS PAGE. The phosphotyrosine level on Tie2 protein were then determined by a-phosphotyrosine antibodies (4G10, Upstate Biotechnology) on Western blots. Films were scanned and % inhibition as compared to untreated control was determined."

EQUIVALENTS

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.